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**13. ABSTRACT** ***(Maximum 200 Words)***

The ultimate goal of this project is to develop immunotherapeutic agents that target a patient's own T cells against their cancer. The project took two directions to accomplish this goal: the development of novel single-chain bispecific antibodies and new animal models that more closely resemble human cancers. Although the project has seen some changes in the original plans (such as the focus on a different tumor antigen, the folate receptor), significant progress was made in understanding the effects of this class of immunotherapeutics. In addition, we have developed and now focus on two animal models that provide realistic, albeit challenging, opportunities to evaluate such agents. Our primary findings are that: 1) small, bispecific antibodies with conjugated folate can target folate-receptor-positive tumor cells, both *in vitro* and *in vivo*; 2) growth of established transplanted human tumors in TCR/RAG$^{-/-}$ mice and endogneous brain tumors in SV40-transgenic mice can be slowed but could not be cured with bispecific conjugates alone; T cell infiltration into the tumors can be achieved but this infiltration needs to be sustained, and 3) CD28 agonists and CTLA-4 antagonists can be used to sustain T cell activity and an anti-CTLA-4 single-chain antibody was engineered for this purpose.

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## INTRODUCTION
**(Adapted in part from original proposal and from previous annual reports)**

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2. This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (1). A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (e.g. (2-6)). In fact, the past two years has seen one of the significant advances in breast cancer treatment with successful clinical trials of the Genentech anti-erb-B2 antibody "Herceptin" (7, 8).

Other anti-erb-B2 agents that are at earlier stages of development include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through T cell effectors. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose of this project has been twofold. First, to construct novel bispecific antibodies that will show optimal tumor targeting potential. Second, to develop *in vivo* animal models that will most closely resemble human cancer and its treatment. The animal models should allow the testing of the bispecific agents in comparison with other conventional bispecific antibodies. There are many

potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed.

The original four specific aims of this project were: 1) Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$); 2) To use a simple screening method to search for agents that increase the sensitivity of erbB-$2^+$ breast cancer cell lines to lysis by CTL; 3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, $RAG^{-/-}$); and 4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/$RAG^{-/-}$ human xenograft system.

Since the submission of this grant application there have been a number of findings that have impacted the direction of the project. Perhaps chief among these is the basic understanding of how T cells are activated and inactivated (9-12). Understanding these processes are key to the rational development of bispecific antibodies that control and redirect the activity of T cells. It is especially relevant to this project that the activation of T cells is now known to be a complex process involving the triggering of multiple surface molecules (Figure 1, next page). It is clear that at least two signals must be received by the T cell: Signal 1 through the T cell receptor/CD3 complex and Signal 2 through the co-stimulatory molecule CD28 (which binds to the ligand B7).

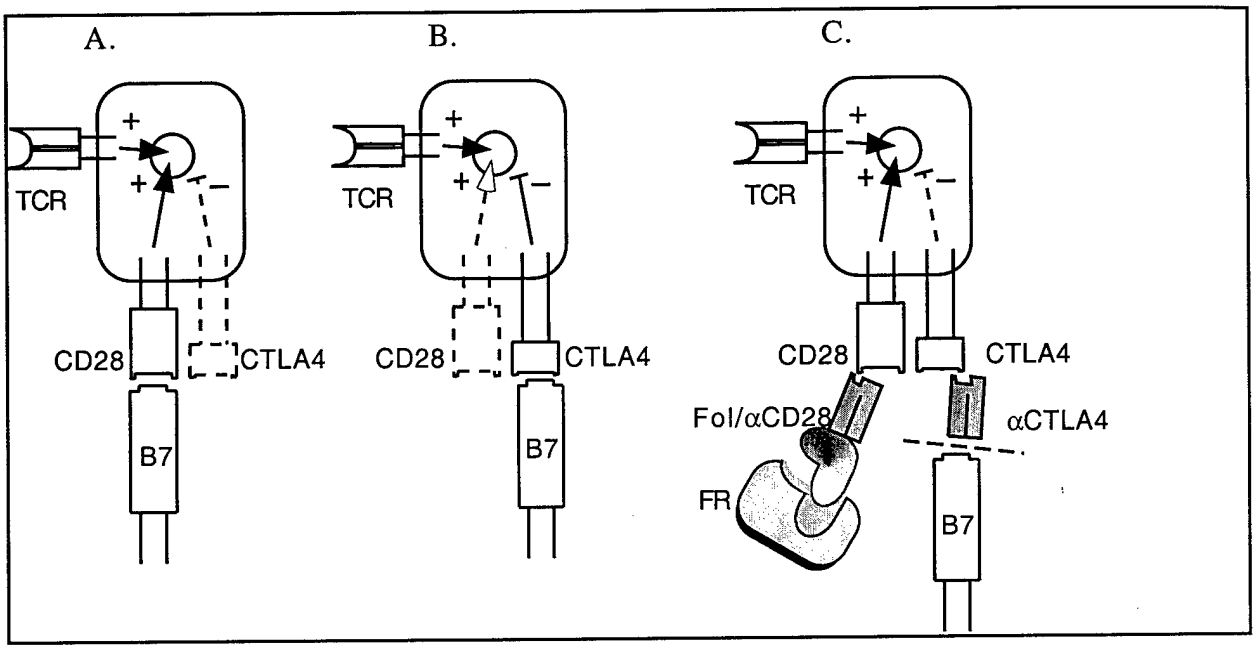
There has also been considerable interest in the past two years in the molecule CTLA-4, present on the surface of activated T cells. CTLA-4 functions as a negative regulator of T cells (13, 14) and blocking of its interaction with its ligand B7 leads to sustained T cell activity (see Figure 1, next page). In several studies, treatment with anti-CTLA-4 antibodies led to T cell-dependent elimination of the tumors (15-17). Given this complexity, it has become even more apparent to us that the successful application of bispecific antibodies in breast cancer therapy will require the use of defined animal models. These will be needed to evaluate not only the various agents that redirect the activity of T cells to tumors (as described in the original grant application) but to evaluate the various agents that might lead to sustained activity of T cells.

In this report, we summarize our progress toward each of the goals outlined above, focusing on three areas of effort:

- **Production and testing of bispecific antibodies that target tumor cells**
- **Development of animal models for the testing and improvement of bispecific agents that target tumors for destruction by T cells**
- **Engineering and characterization of the anti-CTLA-4 antibody 4F10.**

Accomplishments toward these three goals are provided in the body of this report. This is followed by the status of Tasks outlined in the approved Statement of Work from the latest annual report. We cite throughout the report, within the three areas referred to above, publications that were made possible by support from this award. Reprints of these papers are provided in the Appendix.

**Figure 1. Diagram of the positive or negative signals induced by multivalent cross-linking of CD28 or CTLA4, respectively, and monovalent inhibition of CTLA4.** **A.** Multivalent cross-linking of CD28 through B7 on APC leads to enhanced T cell proliferation. **B.** Multivalent cross-linking of CTLA4 through B7 on APC leads to inhibition of T cell proliferation. **C.** Enhanced signalling through FR-mediated cross-linking of CD28 and inhibition of CTLA4 signals through monovalent anti-CTLA4, as proposed in the studies described here.




## BODY
## (Adapted in part from previous annual reports)

### 1) Production and testing of bispecific antibodies that target tumor cells

The original proposal sought to engineer and test a new class of antibodies, the bispecific, single-chain antibody. The possible advantages of this form, compared to conventional bispecific antibodies, are its reduced size and ease of production. Our efforts along these lines followed two directions: A) The cloning and expression of an anti-erb-B2 based bispecific antibodies. B) The discovery of novel folate receptor-based bispecific agents. Results for each of these areas are summarized below.

#### A) The cloning and expression of anti-erb-B2 based bispecific antibodies.

**Cloning of the $V_H$ and $V_L$ genes from the anti-erbB-2 antibody 800E6.** Two different mouse hybridomas (900F4 and 800E6) that secrete antibodies with high affinity for human erbB2 (18) were used to clone a single-chain anti-erbB2 antibody. RNA derived from each hybridoma was isolated and used in the synthesis of cDNA. Degenerate primer pairs for mouse $V_L$ and $J_L$ and $V_H$ and $J_H$ were used to amplify directly from cDNA following a procedure that has been used successfully by our lab with other antibodies. Both hybridomas yielded PCR products

of the expected size (~350 bp) for the $V_H$ region but both hybridomas yielded only a 230 bp product for the $V_L$ region (in contrast to the ~ 350 bp that was expected). It is likely that both antibodies use $V_L$ region genes that were not represented by the degenerate primers. Because we were able to successfully clone the $V_H$ PCR product for 800E6 initially, the light chain from 800E6 was isolated from SDS-PAGE gels in an effort to obtain information about the $V_L$ gene family used by this antibody. The sequence DIVMXQXHKF was identified and this sequence corresponded to the $V_K$ subgroup I family in mice. A primer that corresponded to this sequence was used to successfully amplify a 350 bp PCR product from the 800E6 cDNA.

The $V_H$ and $V_L$ PCR products from 800E6 were cloned sequentially into the single-chain expression vector. The nucleotide sequence of the 800 base pair insert was determined by automated sequencing with primers that flank the scFv gene (Figure 2). Both the $V_L$ and $V_H$ genes

**Figure 2.** Sequence of the anti-erbB2 scFv from hybridoma 800E6.

```
GACGTCGTGATGACTCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGCAAG
 D  V  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S  I  T  C  K

GCCAGTCAGGATGTGGGTACTGCTGTAGCCTGGTATCAACAGAAACCAGGGCAATCTCCTAAACTACTGATT
 A  S  Q  D  V  G  T  A  V  A  W  Y  Q  Q  K  P  G  Q  S  P  K  L  L  I

TACTGGGCATCCACCCGGCACACTGGAGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACT
 Y  W  A  S  T  R  H  T  G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  T

CTCACCATTAGCAATGTGCAGTCTGAAGACTTGGCAGATTATTTCTGTCAGCAATATAGCAGTTATCCGCACG
 L  T  I  S  N  V  Q  S  E  D  L  A  D  Y  F  C  Q  Q  Y  S  S  Y  R  T

TTCGGTGCAGGGACCAAGCTGGAAATAAAACGT
 F  G  A  G  T  K  L  E  I  K  R

TCCTCCGCGGATGATGCTAAGAAGGATGCTGCTAAGAAGGATGATGCTAAGAAAGATGATGCTAAGAAAGAT
 S  S  A  D  D  A  K  K  D  A  A  K  K  D  D  A  K  K  D  D  A  K  K  D

GCATCC
 A  S

CAGGTGCAGCTGCAGCAGTCAGGGGGAGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATATCCTGCAAGGCT
 Q  V  Q  L  Q  Q  S  G  G  E  L  M  K  P  G  A  S  V  K  I  S  C  K  A

ACTGGCTACACATTCAGTAACTACTGGATAGAGTGGGTGAAGCAGAGGCCTGGACATGGCCTTGAGTGGATT
 T  G  Y  T  F  S  N  Y  W  I  E  W  V  K  Q  R  P  G  H  G  L  E  W  I

GGAGAGATTTTACCTGGAAGTGGTAGTACTAACTACAATGAGAAGCTCAAGGGCAAGGCCACATTCACTGCA
 G  E  I  L  P  G  S  G  S  T  N  Y  N  E  K  L  K  G  K  A  T  F  T  A

GACACATCCTCCAACACAGCCTACATGCAACTCAGCAGCCTGACATCTGAGGACTCTGGCGTCTATTACTGT
 D  T  S  S  N  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  G  V  Y  Y  C

GCAAGAGGGGGGGGTAACTACCCGTACTACTTTGACTACTGGGGCCCAGGAACCTCAGTCACCGTCTCCTCA
 A  R  G  G  G  N  Y  P  Y  Y  F  D  Y  W  G  P  G  T  S  V  T  V  S  S

GAACAAAAGCTTATTTCCGAAGAAGATTTG
 E  Q  K  L  I  S  E  E  D  L
```

800E6 $V_L$

LINKER

800E6 $V_H$

C-MYC TAIL

contained all of the conserved residues that are characteristic of Igs. These included the canonical FGXG of $J_L$ and WGXG of $J_H$ and two cysteines that form the basis of the typical Ig fold. The construction was also shown to be expressed at the protein level based on its reactivity with a monoclonal antibody that is specific for the c-myc peptide that was included at the carboxy terminus of the scFv (data not shown).

**Expression and Purification of scFv-800E6.** The anti-erbB2 scFv was expressed in *E. coli* in large scale fermentation, the cells were broken by passage through a microfluidizer, and the inclusion body pellets were solubilized in guanidine. After refolding by dialysis in a Tris-EDTA-arginine buffer (19), scFv was purified by size exclusion through a G200 HPLC column. Samples were analyzed by SDS-PAGE and ELISA with the anti-c-myc antibody (data not shown). The major protein in the crude preparation exhibited a size of 31 kDa, consistent with the predicted size of the scFv-800E6 (33,559 Da). Approximately 75% of this preparation migrated as aggregates in the size exclusion column but a monomer peak of ~30 kDa (fractions 55 to 60) was identified by ELISA with anti-c-myc as the scFv-800E6. The anti-erbB2 scFv monomer could be isolated at ~1 mg/liter of bacterial culture.

**Cell Surface Binding of scFv-800E6 to erbB2.** In order to determine if the scFv-800E6 could bind to erbB2 on the cell surface, two different assays were used. Initially, flow cytometry with the erbB2 positive tumor cell line SKBR3 was performed in the presence of the scFv, followed by anti-c-myc, and then fluorescein-labelled anti-mouse Ig. The result showed that the scFv preparation bound specifically to the SKBR3 cells (data not shown). In order to determine the affinity of the recombinant scFv relative to the intact antibody, a competitive inhibition assay was performed with $^{125}$I-labelled Fab fragments derived from 800E6. SKBR3 cells were incubated with a constant amount of $^{125}$I-Fab in the presence of various concentrations of unlabeled scFv or Fab fragments (Figure 3). The result indicated that the scFv has an affinity that is within two-fold of that of the Fab fragment ($K_D \sim 10^{-9}$ M). In addition, the scFv preparation appears to be predominantly in the properly folded form.

This single-chain gene has now been cloned downstream of the anti-TCR antibodies 1B2 and KJ16 to produce two different $scFv_2$. 1B2 recognizes a clonotypic determinant on the TCR from CTL clone 2C (20) while KJ16 recognizes the Vβ8 chain of clone 2C (21). Both $scFv_2$ contained the 25 residue interchain linker 205 and a 10 residue c-*myc* tail for detection (Figure 4). These two antibodies were constructed in order to evaluate the effect of different antibody domains on folding of the $scFv_2$ and ultimately on their bispecific activity.

**Construction and Expression of $scFv_2$.** To construct the $scFv_2$, the 800E6 scFv was amplified by PCR using a 5' primer which contained the sequence for the 205c' linker, and a 3' primer that included a HindIII site within the c-*myc* region for cloning into the 1B2 scFv vector or the KJ16 scFv vector. The linker/800E6 scFv fragment was cloned between the $1B2V_H$ (or KJ16 $V_H$) and the *c-myc* tail. Inclusion body pellets from cells that contained the $scFv_2$ genes were solubilized in 6M guanidine. To isolate monomeric $scFv_2$ from proteins which remained aggregated in the presence of guanidine, the solubilized inclusion body was subjected to Superdex G-200 size exclusion in 4M guanidine. The 60 kDa peak fractions were individually refolded by dialysis into Tris-Arginine buffer, and analyzed by SDS-PAGE (data not shown). Fractions with the highest fraction of homogeneous $scFv_2$ were pooled, dialysed against buffer and monomeric $scFv_2$ was isolated by non-denaturing size exclusion chromatography. The peak that represented monomer had a 60kDa apparent molecular weight under reducing and non-reducing conditions (data not shown). As expected, this was approximately twice the size of either the 1B2, KJ16, or 800E6 scFv (30kDa).

**Figure 3.** Comparison of erbB2 binding by 800E6 Fab fragments and scFv. $^{125}$I-Fab fragments were incubated with the erbB2 positive tumor cell SKBR3 in the presence of the indicated concentration of inhibitor. After 1 hour on ice, the mixture was centrifuged through a layer of oil to separate bound and free $^{125}$-I-Fab.

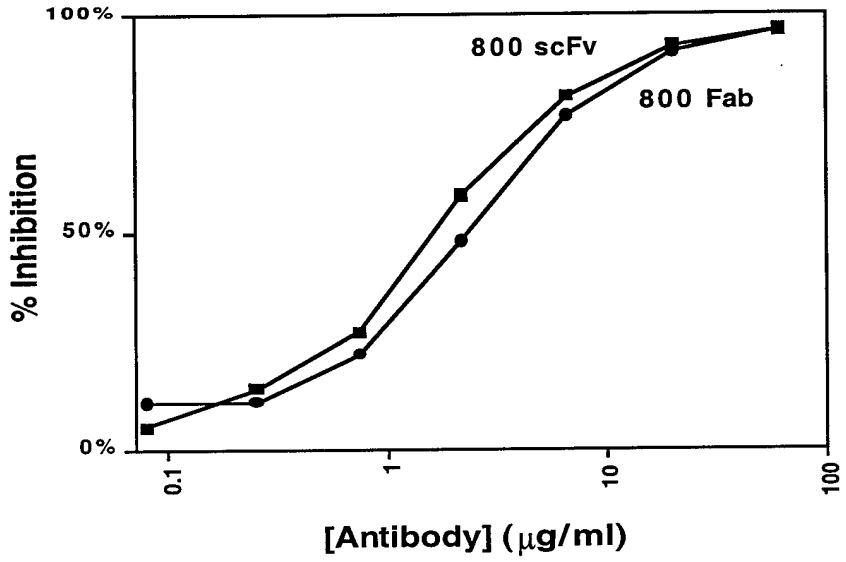
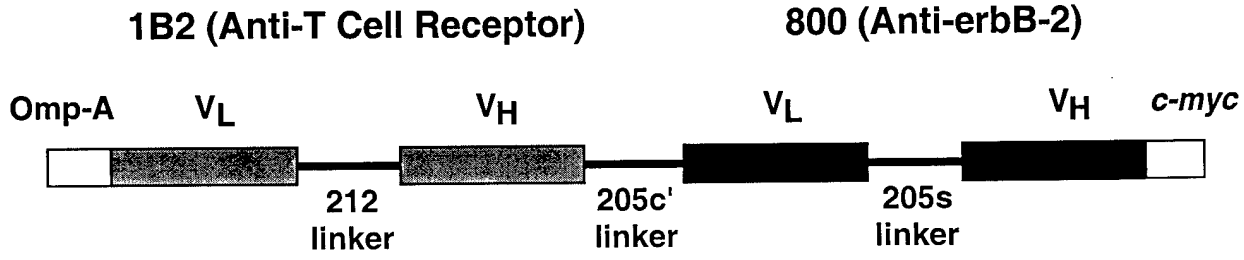


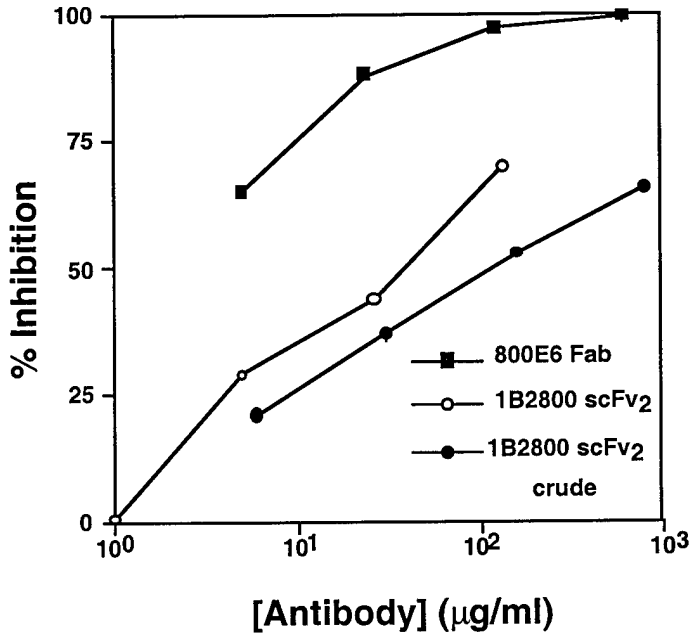

**Figure 4. Schematic of scFv$_2$ gene for single-chain bispecific anti-TCR/anti-erbB-2 antibodies.**



**Binding Properties of Anti-TCR/erbB-2 scFv$_2$ Antibodies.** To examine binding of the scFv$_2$ to erbB-2 on the surface of human breast cancer cells, a competition assay was performed with $^{125}$I-labeled 800E6 Fab fragments. As shown in Figure 5, crude as well as partially purified preparations of 1B2 scFv$_2$ were able to bind erbB-2 on SKBR-3 cells. In contrast to the monovalent 800E6 scFv which bound erbB-2 at nearly equimolar concentrations as 800E6 Fab fragments, bispecific antibody preparations were 10 to 25-fold less efficient than unlabeled Fab fragments. A possible explanation for the observed reduction in binding is that a significant fraction of the scFv$_2$ preparation is not properly folded.

The KJ16/800E6 scFv$_2$ was also examined in an ELISA for binding to soluble T cell receptor absorbed to 96-well plates (data not shown). This antibody (but not the 1B2 scFv$_2$, data not shown) was found to bind specifically to the soluble TCR and to the cell surface of CTL 2C by flow cytometry (data not shown). At this time it is unclear why detectable binding of the 1B2 scFv$_2$ was not detected, although it is likely due to the less efficient folding of this scFv domain compared to the KJ16 scFv domain. In addition, the KJ16/800E6 scFv$_2$ bound to erbB-2+ tumor cell line, BT474 as judged by flow cytometry (data not shown).

**Figure 5. Binding of 1B2/800E6 scFv$_2$ erbB2 on the human tumor line SKBR-3.** HPLC purified scFv$_2$ or crude scFv$_2$ were compared with unlabeled Fab fragments for their ability to inhibit binding of $^{125}$I-labeled 800E6 Fab fragments.




**Bispecific Antibody Activity in CTL-Mediated Killing Assays.** As the scFv$_2$ had erbB-2 binding activity, it was of interest to examine if the antibodies could redirect CTL 2C to lyse target cells. This was of particular interest because no one has yet reported redirected killing with an anti-erbB-2 scFv$_2$ and it was possible that this property could be influenced by the nature of the inter-scFv linker. As expected, the L$^d$ alloreactive CTL 2C was unable to lyse human,

erbB-2 positive breast carcinoma line BT-474 without the bispecific antibody. However, BT-474 were lysed when 1B2/800E6 $scFv_2$ was added to the CTL/target cell assay (Table 1). The observed lysis was dependent on the concentration of bispecific antibody (Table 1, Exp. 2), and could be inhibited with monospecific 1B2 or 800 intact Ab (Table 1, Exp. 1). The ability to mediate lysis requires covalent linkage of the two antibody binding domains as evidenced by the inability of a mixture of 1B2 and 800E6 monospecific antibodies to redirect lysis (Table 1, Exp.1).

**Table 1. Redirected lysis of erbB-2+ tumor cells by the 1B2/800E6 $scFv_2$.**

| Experiment 1 | antibody | µg/ml | Inhibitor | % Specific $^{51}$Cr Release BT-474 |
|---|---|---|---|---|
| | - | - | - | 0 |
| | 1B2 Ig + 800E6 Ig | 25 + 25 | - | 4 |
| | $scFv_2$ | 13 | - | 22 |
| | $scFv_2$ | 13 | 25 µg/ml 1B2 Ig | 0 |
| | $scFv_2$ | 13 | 25 µg/ml 800E6 Ig | 0 |
| | $scFv_2$ | 13 | - | 0* |

| Experiment 2 | antibody | µg/ml | % Specific $^{51}$Cr Release BT-474 |
|---|---|---|---|
| | $scFv_2$ | 5 | 20 |
| | $scFv_2$ | 2.5 | 21 |
| | $scFv_2$ | 1.3 | 18 |
| | $scFv_2$ | 0.6 | 6 |

*In this assay, target cells were added to wells with media and $scFv_2$, without effector cells. In all other wells 2C CTLs were added at an effector to target cell ratio of 10:1. All assays were performed in triplicate and standard error was less than ± 3%.

As described in last year's report, we elected not to continue our efforts in improving the folding efficiency and potency of the anti-erbB2 antibodies because we believed that our efforts wee better spent on other activities (Sections 2 and 3 below). This was based in large part on the increased activity on anti-erbB2 antibodies from companies that focus on protein engineering. Thus, we proposed that it would be wiser for our lab to focus on the general principles that will guide the optimal use of these agents in redirecting the activities of T cells against breast cancer. For example, companies tend to devote fewer resources toward improving animal models for comparing such products. Improved animal models can be extremely useful in that they guide the direction of expensive clinical trials only for the most promising agents.

**Publications:**

Kranz, D. M., M. Gruber, and E. R. Wilson (1995) Properties of Bispecific Single Chain Antibodies Expressed in *E. coli*. *J. Hematotherapy*. 4:403-408.

### B) The discovery of novel folate receptor-based bispecific agents.

Our lab has been simultaneously developing a new class of bispecific agents that have the same purpose as the anti-erbB-2 conjugates (i.e. to target tumor cells for lysis by T cells). These bispecific agents are conjugates of the small molecule folate (440 daltons) and an anti-TCR antibody (22, 23). A high-affinity folate receptor (FR) has been shown to be expressed on most ovarian and many breast cancer tumors (e.g. (24)). In fact, the first generation of anti-FR/anti-CD3 antibodies continue to be tested in clinical trials for ovarian cancer (25-27). We have shown that the folate/antibody agents have potent tumor targeting activity, either as folate/IgG, folate/Fab, or folate/scFv conjugates. Thus, the folate conjugates provide a useful system to begin to evaluate various aspects of the TCR/RAG model. It is important to realize that these experiments are directly applicable to this project, as the findings will impact how erbB-2-specific antibodies can be used to optimally recruit and redirect T cells.

A complete presentation of our results with folate/antibody conjugates are presented in the reprints provided in the addendix. The work describes three phases:

- Description of the synthesis and testing of three different folate/anti-TCR antibody conjugates against mouse tumor lines (Kranz, D.M., T. A. Patrick, K. E. Brigle, M. J. Spinella, and E. J. Roy (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate Receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* 92:9057-9061). This paper shows that folate can be attached to the antibodies using a carbodiimide reaction, yielding folate conjugates that redirect the lysis of folate-receptor-positive tumors by T cells. The conjugates are as potent as any of the larger, conventional bispecific antibodies produced previously. A patent application was also filed on this finding and issued in 1996 (U.S. patent #5,547,668; Aug. 20, 1996; Conjugates of folate anti-effector cell antibodies).

- Description of the synthesis and testing of an even smaller bispecific agent, folate conjugated to a single-chain anti-TCR antibody (Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346). This paper shows that folate can be attached to a 30 KDa single-chain antibody produced in *E. coli* and that the folate/scFv conjugates are as potent as the folate/IgG conjugates.

- Description of folate conjugate use in mediating the lysis of human tumor targets (Rund, L.A., B.K. Cho, T.C. Manning, P.D. Holler, E. J. Roy, and D.M. Kranz (1999) Bispecific Agents Target Endogenous Murine T Cells Against Human Tumor Xenografts. *Int. J. Cancer* 83:141-149). This paper shows that the folate conjugates described above are efficient in redirecting T cell activity against human tumors, both *in vitro* and *in vivo* (see topic 2A below).

### Publications:

Kranz, D.M., T. A. Patrick, K. E. Brigle, M. J. Spinella, and E. J. Roy (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate Receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* 92:9057-9061.

Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346.

Rund, L.A., B.K. Cho, T.C. Manning, P.D. Holler, E. J. Roy, and D.M. Kranz (1999) Bispecific Agents Target Endogenous Murine T Cells Against Human Tumor Xenografts. *Int. J. Cancer* 83:141-149.

2) **Development of animal models for the testing and improvement of bispecific agents that target tumors for destruction by T cells.** Two different animal models for evaluating bispecific antibodies were developed: A) TCR/RAG-/- mice and B) SV40 Transgenic Mice that Develop Endogenous Tumors of the Choroid Plexus.

A) **TCR/RAG-/- mice.** A complete presentation of our results on the development of the 2C T cell receptor transgenic mice, crossed and back-crossed to recombination-activating-gene knockout mice (TCR/RAG$^{-/-}$) are presented in the reprints provided in the addendix. This work describes three phases:

- Description of the production and screening methods associated with the TCR/RAG$^{-/-}$ mice colony. (Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1998) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release.* 53: 77-84). This paper describes the rationale for producing the TCR/RAG$^{-/-}$ mice and the assays used to screen mice.

- Description of the lymphoid phenotype of the mice and their ability to eliminate large tumor burdens that bear the appropriate peptide>MHC antigen (Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in CD8$^{+}$ TCR Transgenic/RAG$^{-/-}$ Mice. *J. Immunol.* 159:4665-4675). This paper characterized the peripheral lymphoid organs of the mice as containing a monoclonal population of CD8$^{+}$ T cells, it showed these cells were capable of full antigen-dependent activation, and it showed that this population of T cells was cable of eliminating a large transplanted tumor of >$10^{8}$ cells).

- Demonstration that the TCR/RAG$^{-/-}$ mice were capable of accepting transplants of human tumor cells, including the folate-receptor-positive line KB and human breast cancer lines and that bispecific antibodies could be used to mediate the rejection of the transplanted human tumors (Rund, L.A., B.K. Cho, T.C. Manning, P.D. Holler, E. J. Roy, and D.M. Kranz (1999) Bispecific Agents Target Endogenous Murine T Cells Against Human Tumor Xenografts. *Int. J. Cancer* 83:141-149). This paper is the first study to show that a human tumor can be transplanted into a T-cell-competent animal model. It was also the first to show that folate/anti-TCR antibody conjugates could be used to redirect the activity of T cells *in vivo*. The study also showed that it was not possible to use systemic administration of the bispecific antibodies to achieve adequate elimination of well-established tumors. Thus, we have explored the use of additional strategies to sustain the activity of T cells against such tumors. The SV40 tumor model described below has facilitated our efforts to design and test improved agents in this regard.

## Publications

Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* 159:4665-4675.

Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1998) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release.* 53: 77-84.

Rund, L.A., B.K. Cho, T.C. Manning, P.D. Holler, E. J. Roy, and D.M. Kranz (1999) Bispecific Agents Target Endogenous Murine T Cells Against Human Tumor Xenografts. *Int. J. Cancer* 83:141-149.

B) **SV40 Transgenic Mice that Develop Endogenous Tumors of the Choroid Plexus.** As a model of an endogenously arising tumor in an immunocompetent host, we chose SV11 mice that are transgenic for the SV40 large T antigen and develop choroid plexus tumors (28-31). The etiology of SV11 tumors (i.e. SV40 large T antigen interferes with p53 and pRB) may mimic that of some pediatric brain tumors. SV40 has been isolated from pediatric choroid plexus tumors and ependymomas (32, 33) (the significance of finding SV40 in these tumors and other types of human tumors is still being debated (34)). In addition, a high affinity folate receptor has been identified on pediatric choroid plexus tumors and ependymomas (35). These high affinity folate receptors are present on most ovarian cancers and some breast cancers (24). The SV11 strain of mice was originally reported to have a mean survival age of 104 days. Our colony of SV11 mice, now over 30 generations later, has a nearly identical survival curve, with mean survival times of 100-105 days (for various cohorts).

A complete presentation of our results on the SV40-transgenic model are presented in the reprints provided in the addendix and focused on the following two areas:

- Detection and characterization of the folate-receptor (FR) as a tumor –associated target on endogenously arising tumors (Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123). Because human choroid plexus tumors express high levels of FR, we evaluated endogenously arising brain tumors of the SV40 transgenic mice for the expression the high affinity folate receptor. The FR were characterized by several approaches (e.g. Western Blotting, RT-PCR, immunohistochemistry, and $^{125}$I-folate binding assays). We found that the tumors express FR with properties that are very similar to the human receptor. These properties included its molecular weight (~38 kDa), size of transcripts, and a binding affinity ($K_D$) for folate of ~ 1 nM. Flow cytometry of FR on the tumor cells and immunohistochemistry indicated that virtually all of the viable cells are FR positive.

- Description of the ability of bispecific conjugates to redirect the activity of T cells against the SV40-induced tumors (Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick, and D. M. Kranz (1998) Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. *Int. J. Cancer.* 76: 761-766; Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy

Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* 78: 470-479). These papers describe the procedures and effects of folate/anti-TCR antibody conjugates in recruiting t cells to an established tumor. Although significant increases in animal survival were achieved, the results were consistent with those in the TCR/RAG-/- mice in that it will be necessary to sustain a T cell response against established tumors in order to optimize the effectiveness of redirecting T cell activity. The final section of this report (Section 3 below) describes our efforts toward using monovalent anti-CTLA-4 antibodies for this purpose.

**Publications:**

Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick, and D. M. Kranz (1998) Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. *Int. J. Cancer.* 76: 761-766.

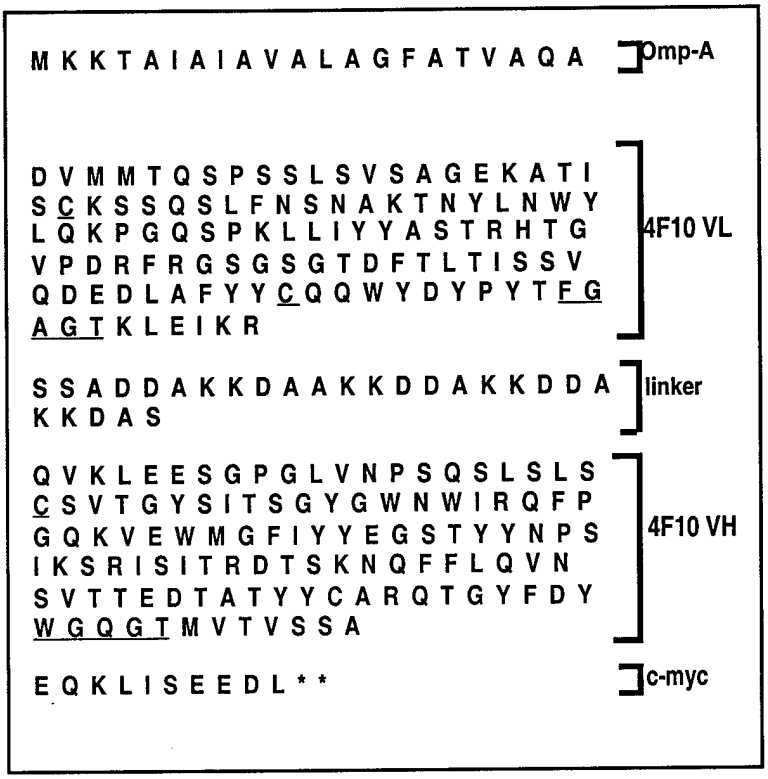
Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* 78: 470-479.

## 3) Engineering and characterization of the anti-CTLA-4 antibody 4F10.

It has been shown that anti-CTLA4 antibodies are capable of blocking the inactivation of T cells and that they can thereby enhance anti-tumor activity (15-17, 36, 37). However, intact anti-CTLA4 antibodies can also inactivate T cells, if the antibody is presented in multivalent form by FcR bearing cells (38). To eliminate this latter possibility, our lab has begun a collaboration with Jeff Bluestone to engineer monovalent scFv of the anti-CTLA4 antibody 4F10. The monovalent scFv and Fab fragments of the antibody have been used in an attempt to sustain the activity of T cells. In addition, the scFv has now been used to engineer more potent forms of the antibody through directed evolution (see below)

To clone and express the scFv of the anti-CTLA4 antibody 4F10, $V_H$ and $V_L$ genes were amplified from hybridoma cDNA with degenerate primers. $V_H$, $J_H$ , $V_L$, and $J_L$ primers were based on the alignment of several published hamster V regions (39, 40). PCR products were cloned into a temperature inducible *E. coli* expression plasmid, behind the ompA signal sequence. This scFv expression vector also contains a ten residue c-*myc* region at the carboxy terminus to allow analysis of total protein expression using anti-c-*myc* antibodies in an ELISA and binding to T cells by flow cytometry. The $V_H$ and $V_L$ sequences were in frame and they conformed to the expected Ig-like domains, incuding invariant cysteine residues and the FGXGT and WGXGT of $J_L$ and $J_H$ regions (Figure 6). In addition, the $V_L$ chain sequence was consistent with amino terminal a. a. sequence (DIMMTQSPSS, where the primer only extended through the Q) obtained from the 4F10 light chain.

**Figure 6. Sequence of anti-CTLA-4 scFv-4F10.**




Inclusion bodies of the scFv-4F10 were solubilized in 6M guanidine in 100 mM Tris, 2mM EDTA, pH 8, and refolded by dialysis into a Tris/Arginine buffer or diluted 100 fold into a Tris buffer. A 30 kDa scFv protein (Figure 7) was detectable by ELISA and Western blots with anti-c-*myc* antibodies (data not shown). Although both refolding methods yielded detectable protein, the dilution method followed by concentration of the sample was found to yield the most active preparations. Because yields using this method have been uniformly low (100 µg/liter of culture), other refolding conditions are currently being tested. In addition, a 6His tail was cloned at the 3' end of the scFv for purification over a Ni column. Considerably more scFv protein has recently been obtained using this method (Figure 7), but this protein proved to be largely inactive (data not shown).

Unlabeled Fab fragments from 4F10 and the scFv-4F10 were compared by saturation and competition binding experiments with $^{125}$I-labeled Fab fragments. For these experiments, CHO cells transfected with the CTLA4 gene (obtained from Jeff Bluestone) were used in an oil spin assay. As shown in Figure 8, the Fab fragments exhibited a $K_D$ = 98 nM. Partially purified and refolded scFv showed complete inhibition of binding by $^{125}$I-Fab fragments. In addition, binding of the crude refolded scFv to CTLA4 transfected CHO cells was detected with anti-c-*myc* antibodies (data not shown). Efforts to purify larger quantities showed that this scFv is refolded at very low yields (< 100 µg/liter). Efforts to improve the scFv to produce larger quantities are described below.

**Figure 7. SDS-PAGE gel of the single-chain Fv of anti-CTLA4 antibody 4F10.** The scFv 4F10, with or without a hexahistidine tail, was overexpressed in *E. coli*. Cells were lysed by microfluidizing, the isolated inclusion bodies were solubilized in 6M guanidine in 100 mM Tris, 2 mM EDTA, pH 8.0, and refolded by dialysis into Tris buffer containing 0.4 M arginine. After electrophoresis, he gel was stained with Coomasie blue.

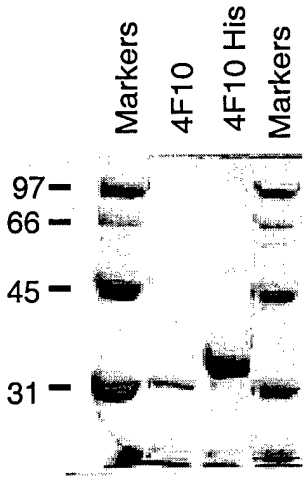
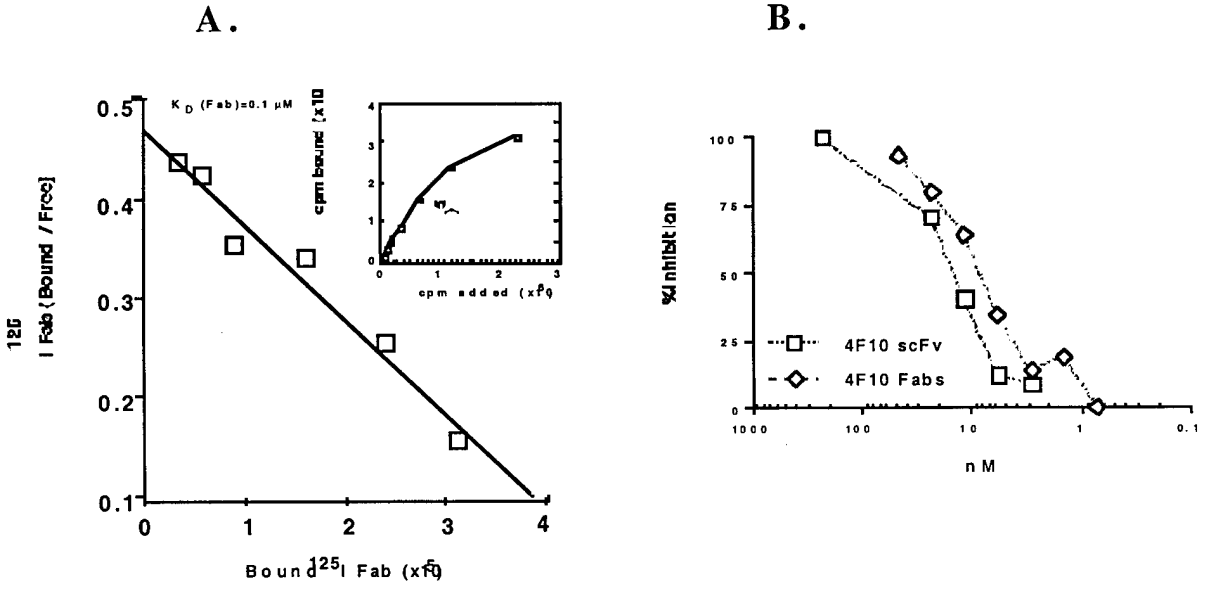



**Figure 8. Binding of anti-CTLA4 antibody 4F10 Fab fragments and single-chain Fv fragments to CTLA4.**

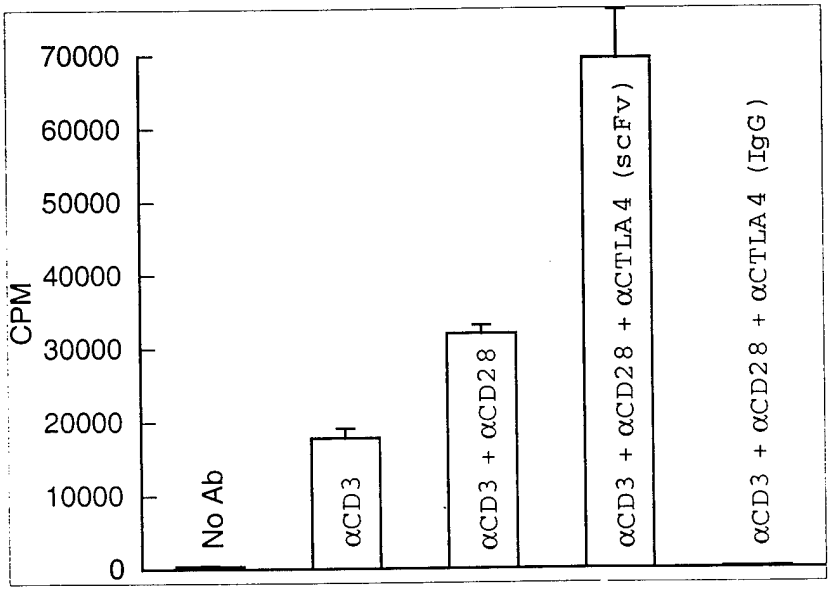
**(A) Binding of $^{125}$I-4F10 Fab Fragment.** Various concentrations of the iodinated-4F10 Fab fragments were added to Chinese Hamster Ovary (CHO) cells transfected with the CTLA4 gene (CTLA4 CHO). After 30 min on ice, bound ligand was separated from free ligand by centrifugation through dibutyl phthalate/olive oil, and pelleted cells were counted in a gamma counter.

**(B) Competitive Binding by scFv 4F10.** CTLA4 CHO cells were incubated with $^{125}$I-labeled 4F10 Fab fragments and serial dilutions of unlabeled 4F10 Fab fragments or 4F10 scFv fragments for 1 hr on ice. Bound and free were separated by centrifugation of the cells through oil as above. Percentage inhibition was caculated as ((cpm bound with no inhibitor) - (cpm bound with inhibitor)) x 100)/(cpm bound with no inhibitor).



A negative proliferative signal can be elicited by multivalent cross-linking of CTLA-4. This cross-linking can be achieved either by the CTLA4 interaction with B7 on APCs or by the CTLA4 interaction with anti-CTLA4-IgG presented by FcR-bearing cells (see Figure 1). Monovalent anti-CTLA4 (scFv or Fab) can prevent the CTL4:B7 interaction and thereby allow optimal positive signaling generated through CD28 (Figure 1C). We have recently confirmed the hypothesis that a single chain form of anti-CTLA4 will enhance T cell activation, as evidenced in a proliferation assay (Figure 9). Figure 9 illustrates that anti-CD28 combined with anti-CD3 induces proliferation of a purified T cell preparation from C57 mice. Anti-CTLA4 scFv further increased this proliferation. As noted above, the IgG form of anti-CTLA4 can induce the opposite effect (i.e. reducing T cell proliferation). This latter result is presumed to be due to cross-linking of the anti-CTLA4 by FcR-bearing cells. This finding illustrates the difference between crosslinking and blocking antibodies, and provides the rationale for why it would be counter productive to folate conjugate the anti-CTLA4 scFv antibody.

**Figure 9. Enhancement of T cell proliferation with monovalent anti-CTLA-4 antibodies.** Splenocytes were isolated using a Variomacs magnetic cell sorter using anti-Thy 1.2, and 2 x $10^5$ cells were cultured in 96 well plates for 60 hrs in the presence of indicated antibodies (0.01 μg/ml anti-CD3, 0.1 μg/ml anti-CD28, 10 μg/ml scFV anti-CTLA4, 10μg/ml IgG anti-CTLA4), following by 12 hrs of incorporation of $^3$H-thymidine. Cpm are means + SEM of triplicate determinations. A titration of antibody concentrations produced similar results (not shown).

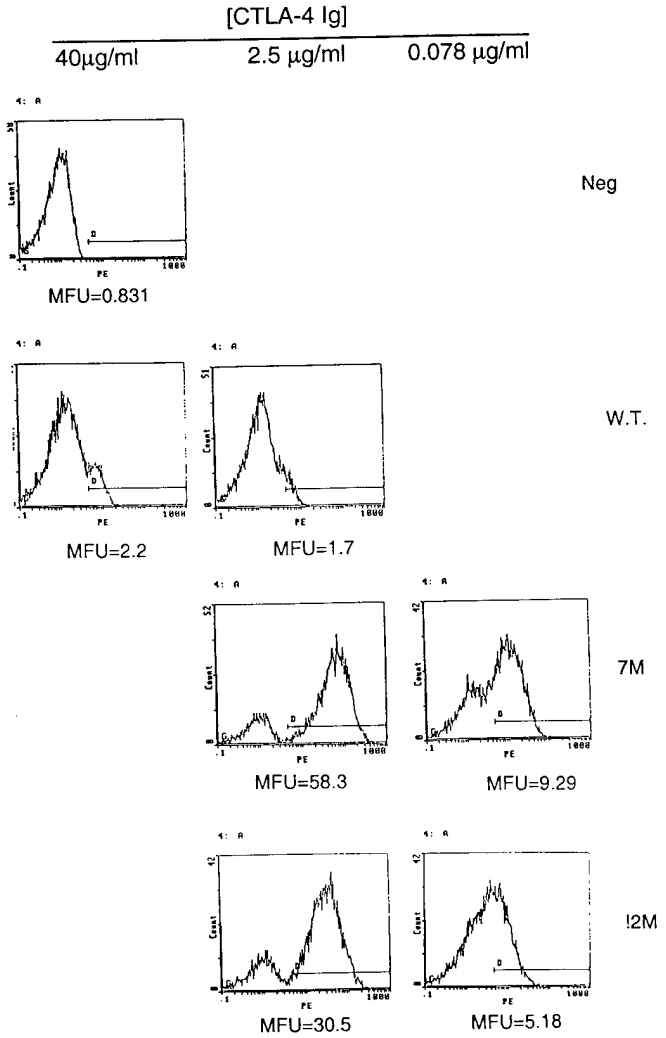



The 4F10 scFv has proven to yield lower amounts of refolded antibody than many of the other scFv our lab has worked with (data not shown). In our experience, this is likely due to $V_L$ and/or $V_H$ regions that are less stable. We have successfully improved the stability of scTCR using the yeast display system for directed evolution (41). In addition, the yeast system has been used to increase the affinity of scFv domains (42, 43). Both stability and affinity increases are likely to improve the *in vivo* effectiveness of the anti-CTLA-4 scFv and thereby its ability to better sustain T cell activity.

To approach this with the scFv-4F10, the gene was cloned into the yeast display vector that contains the Aga-2 gene as an amino terminal fusion (42). After cloning, the plasmid was transformed into yeast and the cells were induced for protein expression. As shown in Figure 10, the wild-type scFv-4F10 was detected at very low levels on the surface of the yeast and only at the highest concentration of labeled-CTLA-4Ig (40μg/ml). This finding was consistent with the low yields that are obtained with refolding from *E. coli* inclusion bodies.

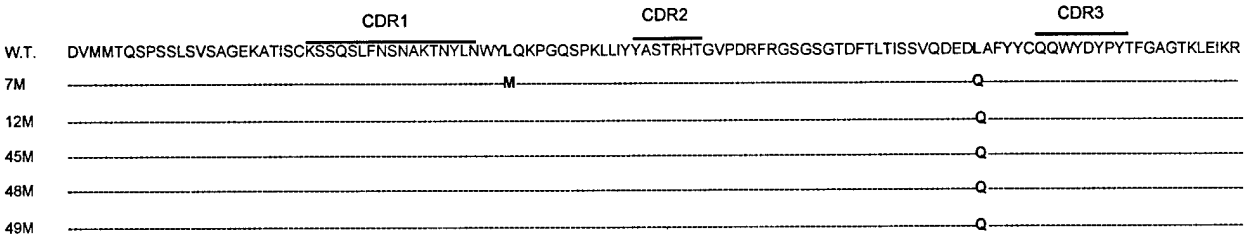
To generate stabilized scFv-4F10 mutants, the entire scFv gene was mutagenized using error-prone PCR and the product was subcloned into the display vector. A library of approximately $10^5$ yeast cells bearing the mutated scFv genes was then subjected to three rounds of sorting by flow cytometry, using the CTLA-4Ig at limiting concentrations. Several mutant colonies were isolated and retested for staining with the CTLA-4Ig. As shown in Figure 10, two of these mutants (7M and 12M) were detected at the lowest concentrations of CTLA-4 tested (2.5 and 0.078 μg/ml).

**Figure 10. Flow cytometry profiles of yeast displayed anti-CTLA4-scFv wild type and two mutants.** Yeast cells that express the Aga-2/scFv were incubated with CTLA-4Ig for 1 hour on ice, followed by biotinylated rabbit anti-mouse IgG2a, and then strepavidin-phycoerythrin.



Plasmids from several mutant colonies were rescued and subjected to DNA sequencing. Among five mutants that were sequenced, there were two unique sequences (Figure 11). Mutant 7M contained two mutations in framework regions of the $V_L$ gene and the others mutants (e.g. 12M) contained a single mutation in the framework region of the $V_L$. These mutations likely act by increasing the stability of the scFv-4F10, since they are located outside the binding site CDRs. We have observed similar mutations increased the surface expression, stability, and production levels of a scTCR. Future studies will involve further mutation and selections to isolate higher affinity anti-CTLA4 scFv, using the 7M gene as a scaffold.

**Figure 11. Sequences of anti-CTLA-4 scFv 4F10 wild-type and mutants isolated by yeast display selections.**




## Statement of Work: Final Status

**Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12. Completed, as described in Section 1 above.

**Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12. Completed. These findings and results of Task 5 were described in an earlier Annual Report and are not included in this report in order to focus on our more important findings.

**Task 3,** Breeding of transgenic TCR/RAG$^{-/-}$ mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period. Completed.

**Task 4,** Cloning, expression, and *in vitro* testing of bispecific single-chain $scFv_2$ antibody (1B2/erbB-2), months 13-24. Completed. Antibody proved to be poorly folded and effort was focused on other activities.

**Task 5,** Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN-$\gamma$, TNF-$\alpha$, estrogen, tamoxifen, months 13-30. Completed. Tumor lines proved to be more susceptible after growth in IFN-$\gamma$ and TNF-$\alpha$.

**Task 6,** Transplantation of various erbB-2$^{+}$ tumor cell lines into TCR/RAG$^{-/-}$ mice and evaluation of tumor incidence, months 16-36. Completed.

**Task 7,** Purification and *in vitro* testing of bispecific $Fab_2$ antibody (1B2/erbB-2), months 30-48. Did not complete, in order to focus efforts on anti-CTLA-4 studies (see Task 11).

**Task 8,** *In vivo* testing of bispecific antibodies in TCR/$RAG^{-/-}$ mice that have received human tumor transplants, months 30-48. This task was modified to include our efforts with the human KB tumor model and folate/antibody conjugates. Completed.

**Task 9.** Characterization of the KJ16/800E6 (anti-Vβ8/anti-erbB2) bispecific $scFv_2$, months 30-48. Completed. Antibody proved to be poorly folded and effort was focused on other activities.

**Task 10.** Expression of $scFv_2$ (1B2/800E6 and KJ16/800E6) in a yeast secretion system, months 30-48. Did not complete, in order to focus efforts on anti-CTLA-4 studies (Task 11).

**Task 11.** Comparison of *in vivo* activation strategies in TCR/$RAG^{-/-}$ mice, months 30-48. This task includes our studies with anti-CD28 bispecific antibodies and soluble forms of the anti-CTLA-4 Fab fragments to sustain the activity of T cells. Completed the cloning, expression, and initial directed-evolution studies with the anti-CTLA4 scFv-4F10. Continuing efforts with both anti-CD28 bispecific conjugates and the anti-CTLA4 scFv, under support of new NIH grant.

## KEY RESEARCH ACCOMPLISHMENTS

- Designed first single-chain bispecific anti-erbB2 antibody.
- Discovered novel method of preparing bispecific anti-tumor conjugates, by attaching folate to T cell-specific antibodies.
- Developed first immunocompetent transgenic mice (TCR/$RAG^{-/-}$) that can be used for targeting transplanted human tumors.
- Proved that endogenous T cells can be redirected against tumor cells in both the TCR/RAG mouse model or the SV40 transgenic model of endogenous tumors.
- Cloned and expressed first scFv against the T cell regulatory molecule CTLA-4.
- Initiated directed-evolution project, using yeast display system, on the anti-CTLA-4 scFv.

## REPORTABLE OUTCOMES

### Publications:

**Papers that were directly supported by this award (reprints provided as an Appendix):**

1. Kranz, D. M., M. Gruber, and E. R. Wilson (1995) Properties of Bispecific Single Chain Antibodies Expressed in *E. coli*. *J. Hematotherapy*. 4:403-408.

2. Kranz, D.M., T. A. Patrick, K. E. Brigle, M. J. Spinella, and E. J. Roy (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate Receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* 92:9057-9061.

3. Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

4. Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346.

5. Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* 159:4665-4675.

6. Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1998) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release.* 53: 77-84.

7. Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick, and D. M. Kranz (1998) Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. *Int. J. Cancer.* 76: 761-766.

8. Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* 78: 470-479.

9. Rund, L.A., B.K. Cho, T.C. Manning, P.D. Holler, E. J. Roy, and D.M. Kranz (1999) Bispecific Agents Target Endogenous Murine T Cells Against Human Tumor Xenografts. *Int. J. Cancer* 83:141-149.

**Abstracts/Presentations:**

1. Gruber, M. and D. M. Kranz (1995) Susceptibility of breast cancer cells to lysis mediated by a bispecific single-chain antibody and cytotoxic T cells. Fourth International Conference on Bispecific Antibodies and Targeted Cellular Cytotoxicity, Hawk's Cay, Florida.

2. Kranz, D.M., M. Gruber, and E.R. Wilson (1995) Properties of bispecific single-chain antibodies expressed in *E. coli.* Fourth International Conference on Bispecific Antibodies and Targeted Cellular Cytotoxicity, Hawk's Cay, Florida.

3. Wiener, E.C., Patrick, T.A., Roy, E.J., Kranz, D.M., and Lauterbur, P.C. (1995) Tumor targeted MRI contrast agents, Society of Magnetic Resonance, Nice, France

4. Roy, E.J., Patrick, T.A., Van Dyke, T.A., and Kranz, D.M. (1995) Bispecific antibodies that direct T cells against folate receptors: a potential treatment for brain tumors in transgenic mice. Eleventh International Conference on Brain Tumor Research and Therapy, Napa, CA

5. E.J. Roy, B.K. Cho, T.A. Patrick, and D.M. Kranz. (1996) Penetration of T cells into brain tumors in SV11 mice following treatment with a single-chain bispecific antibody/folate conjugate. First Scientific Meeting of the Society for Neuro-Oncology. San Francisco, CA

6. Patrick, T.A., Kranz, D.M., and Roy, E.J. (1996) Immunotherapeutic targeting of CTLs toward murine choroid plexus tumors with a folate/anti-CD3e antibody conjugate. Autumn Immunology Conference, Chicago, IL.

7. Patrick, T. A., Kranz, D.M., and Roy, E.J. (1997) Adoptive immunotherapy of murine choroid plexus tumors with cytotoxic T lymphocytes and an anti-tumor/anti-T cell receptor complex (folate/anti-CD3ε) bispecific antibody conjugate. Society for Neuroscience, New Orleans, LA

8. Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1997) Targeting tumor cells with bispecific antibodies and T Cells. Eighth International Symposium on Recent Advances in Drug Delivery Systems. Salt Lake City, UT.

9. Kranz, D. M., T. C. Manning, L. A. Rund, M.M. Gruber, B. K. Cho, and E. J. Roy (1997) A transgenic animal model for targeting tumor cells with cytotoxic T lymphocytes. U.S. Army "Era of Hope" Meeting on Breast Cancer Research, Washington DC.

10. Roy, E.J., L. A. Rund, and D.M. Kranz (1998) IL-12 prolongs survival in a transgenic mouse model of endogenously arising brain tumors. Society for Neuroscience, Los Angelos, CA

11. Patrick, T.A. and Roy, E.J. (1998) Adoptive immunotherapy of murine choroid plexus tumors with an anti-tumor/anti-T cell receptor (folate/anti-CD3) bispecific conjugate. Student Biomedical Research Forum, Chicago, IL.

12. Kranz, D. M., L. A. Rund, B. K. Cho, and E. J. Roy (1998) Bispecific conjugates for targeting tumor cells with T lymphocytes. Australasian Society for Immunology. Melbourne, Australia.

13. Roy, E.J., Rund, L.A., Williams, R., Orr, B.A., and Kranz, D.M. (1999) Bimodal distribution of responses to IL-12 and possible suppession by TGF-β in a mouse model of brain tumors. Society of Neuro-Oncology, Scottsdale, AZ.

**Patents:**

U. S. patent # 5,547,668; Aug. 20, 1996; Conjugates of folate anti-effector cell antibodies. Inventors: Kranz; David M. (Champaign, IL); Roy; Edward J. (Urbana, IL); Patrick; Todd A. (Urbana, IL). Assignee: The Board of Trustees of The University of Illinois (Urbana, IL).

**Degrees obtained that were supported by this award:**

Meegan Gruber, Ph.D. University of Illinois, 1996
Bryan Cho, Ph.D. University of Illinois, 1997
Todd Patrick, Ph.D. University of Illinois, 1998

**Animal Models:**

Transgenic TCR/RAG$^{-/-}$ mice are now available and have been provided to several labs

**Applied funding, based on work supported by this award:**

We are pleased to report that the NIH has awarded an R01 grant (P.I. Edward Roy with myself as co-P.I.) which will began in early 1999 to continue the studies on bispecific antibody targeting in the folate receptor models. It is our belief that these studies will prove to be of significance in the treatment of breast cancer, using similar bispecific antibody conjugates.

## CONCLUSIONS

The ultimate goal of this project is to develop immunotherapeutic agents that would target a patient's own T cells against their cancer. The project focused on two important directions to accomplish this goal. First to develop novel single-chain bispecific antibodies and second to develop new animal models that more closely resemble human cancers. Although the project has seen some changes in the original plans (such as the focus on a different tumor antigen, the folate receptor), significant progress was made in understanding what are the important aspects of a drug that will optimally accomplish this goal. In addition, we have developed and now focus on two animal models that provide realistic, albeit challenging, opportunities to evaluate such immunotherapeutic agents. Our primary findings are that: 1) small, bispecific antibodies that have folate attached covalently can specifically target folate-receptor-positive tumor cells, both *in vitro* and *in vivo*; 2) established transplanted human tumors in TCR/RAG$^{-/-}$ mice and endogneous brain tumors in SV40-transgenic mice are difficult to cure with bispecific antibody agents alone; although T cell infiltration into the tumors can be achieved, this infiltration needs to be sustained for more successful treatments, and 3) development of agents that sustain such activity through the use of CD28 agonists and CTLA-4 antagonists are possible; an anti-CTLA-4 single-chain antibody was engineered for this purpose and will serve as the focus of future efforts.

**"So what section":** We believe that the information gained from this project will some day yield drugs which can be used to target a patient's own T cells against their cancer. These drugs should have minimal side-effects, compared to chemotherapies, because they will have specificities that target them against only the cancer cells and not the patient's own normal cells.

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## PERSONNEL

**Personnel that received salary from this award:**

David M. Kranz, Principle Investigator
Laurie Rund, Postdoctoral Associate
Phil Holman, Postdoctoral Associate
Meegan Gruber, Graduate Research Assistant
Tom Manning, Graduate Research Assistant
Todd Patrick, Graduate Research Assistant
Ryan Favors, Graduate Research Assistant
Evan Parke, Technician

# Appendix

**Nine reprints of papers that were directly supported by this award are attached:**

1. Kranz, D. M., M. Gruber, and E. R. Wilson (1995) Properties of Bispecific Single Chain Antibodies Expressed in *E. coli*. *J. Hematotherapy*. 4:403-408.

2. Kranz, D.M., T. A. Patrick, K. E. Brigle, M. J. Spinella, and E. J. Roy (1995) Conjugates of Folate and Anti-T Cell Receptor Antibodies Specifically Target Folate Receptor Positive Tumor Cells for Lysis. *Proc. Natl. Acad. Sci. USA* 92:9057-9061.

3. Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

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8. Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* 78: 470-479.

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# Properties of Bispecific Single Chain Antibodies Expressed in *Escherichia coli*

DAVID M. KRANZ, MEEGAN GRUBER, and ERIK R. WILSON

## ABSTRACT

**Single chain bispecific antibodies, in which the genes that encode the $V_H$ and $V_L$ regions are linked in tandem, may offer some advantages over other methods of bispecific antibody preparation. To begin to evaluate the potential of this system, a single chain bispecific antibody ($scFv_2$) that binds to the T cell receptor of a cytotoxic T cell clone and to the hapten fluorescein was constructed. The individual scFv regions were joined by a 25 amino acid linker, and the $scFv_2$ protein was obtained from insoluble inclusion bodies after guanidine solubilization and refolding. Fluorescein-purified $scFv_2$ is active at concentrations of ~10 nM (~1 μg/ml) and above in mediating lysis of fluorescein-coupled tumor cells by the cytotoxic T lymphocytes (CTL). This system has now been used to evaluate various features of the $scFv_2$ approach: (a) affinities of the $scFv_2$ for the TCR and fluorescein, (b) yields of the $scFv_2$ from several different purification schemes, (c) relationship of antigen density (i.e., fluorescein density on tumor cells) to the ability to redirect lysis of the tumor cell, and (d) relationship of $scFv_2$ affinity for the tumor antigen to the ability to redirect lysis. This study was performed by using several analogs of fluorescein for which the $scFv_2$ had different affinities. Results showed that it should be possible to select antibodies with appropriate affinities such that tumor cells with typical antigen densities of $10^4$–$10^5$ molecules per cell will be lysed and normal cells that have lower levels of the antigen will be spared.**

## INTRODUCTION

THREE DIFFERENT GENERAL APPROACHES have been applied to the production of bispecific antibodies (BsAb) (reviewed in 1): (a) secretion from hybrid hybridomas, followed by isolation of the appropriate BsAb from the mixture of "mispaired" immunoglobulins, (b) chemical linkage of two intact or $F(ab')_2$ antibodies with bifunctional cross-linking agents or by disulfide shuffling, and (c) genetic engineering to produce either non-covalently linked BsAb [e.g., diabody (2) and leucine zipper strategies (3)] or covalently linked BsAb [e.g., disulfide linkage of independently expressed antibodies (4) or expression of tandem V regions as a single chain (5)]. One advantage of genetic engineering is the ability to design antibodies that lack C regions and, thus, possible undesirable C region-mediated side effects. Other features, such as increased stability, more rapid and efficient purification strategies, and reduced immunogenicity, can also be engineered in this approach.

In principle, the simplest and currently the smallest version of a BsAb consists of the appropriate $V_H$ and $V_L$ regions from two antibodies linked in frame to form a single chain (5). From a production viewpoint, this construction could represent the ideal form of a BsAb in that it requires fermentation of a single strain and purification of a single species, and it does not involve steps of chemical linkage. Nevertheless, the single chain approach has several significant problems that will need to be overcome. The major problem is associated with refolding of the antibody from insoluble fractions of *Escherichia coli*. Standard refolding procedures generally yield a small

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fraction of protein that has both binding sites folded properly, probably because of the heterologous pairing of V regions (6). Evidence from our laboratory indicates that this property accounts for the relatively high concentration (>1 μg/ml) of the recombinant protein that is required to target tumor cells for lysis by the effector cell.

We have begun to develop an animal model for evaluating various BsAb preparations. This model was developed by selective breeding of RAG-1 knockout mice (7) with transgenic mice that express the α and β T cell receptor (TCR) from the cytotoxic T cell clone 2C (8). The RAG-$1^{-/-}$$tg^{TCR}$ mice are immunodeficient and contain a monoclonal population of cytotoxic T lymphocytes (CTL) that express only the TCR of CTL 2C. Since CTL 2C does not recognize human tumor cells (9), the RAG-$1^{-/-}$$tg^{TCR}$ animals should accept human tumor transplants, yet retain the ability to redirect the lysis of the tumor cells by BsAb and the endogenous CTL. In looking ahead to using this model, we have genetically engineered BsAb that recognize the TCR from CTL 2C. These include the clonotypic antibody 1B2 (5,10) and the anti-Vβ8 antibody KJ16 (11) (Bryan Cho and David M. Kranz, unpublished data). Thus, BsAb prepared with these anti-TCR regions can be compared with those that contain the anti-CD3 antibody 2C11 (3,12) for effectiveness in an animal that will be amenable to the in vivo activation of T cells. In this article, we show further results on the purification and characterization of the active form of a bispecific single chain antibody that consists of the anti-TCR antibody 1B2 and an antifluorescein antibody. In addition, this BsAb was used to explore the role of antibody affinity and antigen density in the targeting of tumor cells.

## MATERIALS AND METHODS

### *Cell lines*

The mouse T lymphoma EL4 and the human B lymphoma Daudi were maintained in RPMI 1640 containing 1.3 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin, streptomycin, 5 mM HEPES, and 10% fetal bovine serum (FBS) (complete media). The mouse CTL clone 2C was maintained in the same medium with the addition of 10% supernatants from concanavalin A-stimulated rat spleen cells and mitomycin C-treated BALB/c spleen cells as stimulator cells (13).

### *Bispecific antibody purification*

A single chain bispecific antibody (scBsAb) that contains the $V_H$ and $V_L$ region genes of the anti-T cell receptor antibody 1B2 and the antifluorescein antibody 4-4-20 was expressed in *E. coli* as previously described (5). Briefly, the insoluble inclusion body pellets were solubilized in 6 M guanidine, followed by dialysis against 100 mM Tris, 2 mM EDTA, and 0.4 M arginine. After dialysis, the sample was applied to a fluorescein affinity column, the column was washed, and specific protein was eluted with either 0.1 M fluorescein (5), 100 mM triethylamine, pH 11.5, or 50 mM $Na_2CO_3$, pH 11.5. Affinity-purified samples eluted with 100 mM triethylamine or 50 mM $Na_2CO_3$, pH 11.5, were neutralized and dialyzed into 50 mM Tris, pH 8.5. The 100 mM triethylamine eluted sample was subjected to anion exchange chromatography through Q-Sepharose and eluted with a 0–1 M NaCl gradient. Fractions were monitored for absorbance at 280 nm, for fluorescein binding activity in an ELISA on wells coated with fluorescein-BSA, and for tumor targeting ability in cytotoxicity assays with CTL 2C (5).

### *Fluorescein compounds*

To measure the affinity of the antifluorescein antibody for fluorescein conjugates, 10 mg of fluorescein isothiocyanate isomers I and II (Molecular Probes, Eugene, OR) was added to 10 mg of NtBOC lysine (Sigma Chemical Co., St. Louis, MO) in 2 ml of 20 mM $KPO_4$, pH 9.0, and incubated overnight at ambient temperature. The conjugates were separated from free fluorescein by thin layer chromatography on silica plates developed in water-saturated methylethylketone. Fluorescein-dihexadecanoyl phosphoethanolamine (Fl-DHPE) was purchased from Molecular Probes. FITC I and FITC II were coupled to the ε-amino group on cell surface proteins by incubation at various concentrations in phosphate-buffered saline, pH 8.5, with ~$10^7$ target cells (unlabeled or $^{51}$Cr-labeled). After 15 min at ambient temperature, cells were washed three times before being used for determination of fluorescein densities or in cytotoxicity assays. Fl-DHPE was inserted into cell membranes after preparing vesicles by sonication. Five micrograms Fl-DHPE/ml PBS was sonicated and added to ~$10^7$ cells for 30 min at 37°C. After three washings, cells were used for determination of fluorescein densities or in cytotoxicity assays.

### *Fluorescein affinity and density measurements*

The affinity of antifluorescein antibody 4-4-20 for FITC-I-lysine, Fl-DHPE, and FITC-II-lysine was determined directly by a fluorescence-quenching assay in which aliquots of the fluorescein conjugate were titrated into a 10 nM solution of antibody (14). The estimated $K_d$s of the $scFv_2$ for the ligands was determined to be 1 nM, 6 nM, and 50 nM, respectively (calculated at ambient temperature). Relative affinities were confirmed by competition ELISAs (data not shown).

The density of fluorescein coupled to tumor cell targets was measured by two independent methods. In the case of FITC-I and Fl-DHPE-labeled cells, $^{125}$I-labeled 4-4-20 antibody was used in saturation binding experiments. The same fluorescein-labeled cells were examined by flow cytometry (Coulter Electronics Epics 752), and the fluorescence was compared with fluorescein-labeled calibration beads (Flow Cytometry Standards Corp, San Juan, Puerto Rico). From the number of accessible epitopes determined by the saturation binding curves, a standard curve with the labeled beads was constructed for each experiment and used to determine the number of fluorescein molecules per cell. This approach allowed determination of FITC-II densities, since saturation was difficult to achieve with the $^{125}$I-4420 because of the lower affinity. This also provided a more rapid and routine analysis of FITC-I and Fl-DHPE densities. It should be noted that the number of accessible sites indicated probably represents an overestimate because at 37°C, during a cytotoxicity assay, the density of fluorescein molecules is reduced as a result of internalization or shedding.

### *Cytotoxicity assays*

Target cells were incubated with 50 $\mu$l (125 $\mu$Ci) of $^{51}$Cr for 1 h at 37°C. After being washed in PBS, cells were coupled with fluorescein as described. Labeled cells were added at $2 \times 10^4$ cells per well of a 96-well plate. BsAb preparations were added in complete media, and CTL clone 2C was added at an effector to target cell (E:T) ratio of 5:1. After 4 h at 37°C, supernatant was removed and the percent specific release was calculated (5).

## RESULTS AND DISCUSSION

### *Purification of single chain antifluorescein/anti-T cell receptor antibodies*

Our previous work with the scBsAb scFv$_2$1B2/4420 showed that purified antibody could be readily obtained from a fluorescein affinity column at a yield of ~1 mg/L of culture (5). The affinity-purified antibody generally exhibited an $IC_{50}$ in tumor targeting assays that was no better than ~1 $\mu$g/ml. This value is at least one to two orders of magnitude higher than one might anticipate in comparison with other forms of BsAb (reviewed in 1). Because the affinity of each monospecific antibody is relatively high ($K_d < 10$ nM) (10,15), the low efficiency of the scFv$_2$ is likely to be due to the presence of a significant fraction of misfolded antibody, even in the affinity-purified preparation.

With this in mind, we attempted to further purify the properly folded, active form of the scFv$_2$ by ion-exchange chromatography of the affinity-purified antibody. Because we were concerned that ligand eluted material would retain bound ligand, we chose to elute under conditions of high pH, which have yielded consistently active antibody. Eluted material was subjected to anion exchange chromatography with Q-Sepharose. The Q-Sepharose profiles of total protein and antifluorescein activity are shown in Figure 1. The ability of various fractions to target fluorescein-labeled tumor cells was also examined. The results of this analysis and other fluorescein-binding studies (data not shown) showed that only approximately 10% of the fluorescein affinity purified scFv$_2$, eluted at either low or high pH, retains fluorescein-binding activity. This result compares with a monospecific scFv, where greater than 30% of the activity is retained (data not shown). Thus, the relatively low $IC_{50}$ values that are observed with this scFv$_2$ can be attributed in part to the reduced stability of the fluorescein-binding domain after elution.

In addition, only a fraction of the protein from the Q-Sepharose column that is capable of binding fluorescein is active in the tumor targeting assay (Fig. 1). This result implies that a significant portion of the BsAb has not refolded the anti-TCR domain and that the relatively low $IC_{50}$ values that are observed with this scFv$_2$ are also attributed in part to the presence of misfolded anti-TCR V regions.

Nevertheless, the fraction (fraction 42) that was most active in tumor targeting showed a 10-fold increase in activity compared with the affinity-purified forms (Fig. 2). This finding suggests that it may be possible to obtain scBsAb with activities that begin to approach conventional BsAb. Strategies that will increase the folding

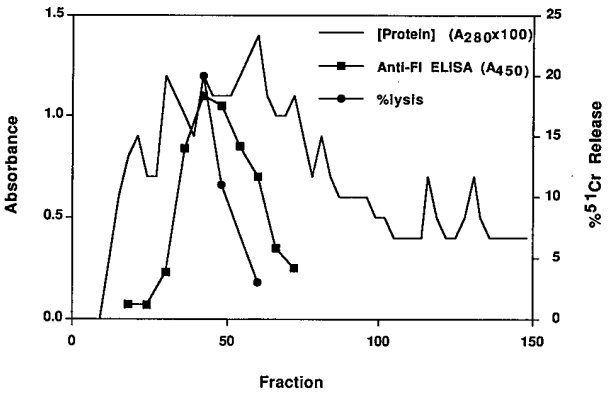


**FIG. 1.** Q-Sepharose column profile of scBsAb 1B2/4420. scFv$_2$ protein eluted from a fluorescein-sepharose column with 100 mM triethylamine was subjected to anion exchange chromatography through Q-Sepharose and eluted with a 0–1M NaCl gradient. Fractions were monitored for absorbance at 280 nm, for fluorescein binding activity in an ELISA on wells coated with fluorescein-BSA, and for tumor targeting ability in cytotoxicity assays with CTL 2C (5).

efficiency of the V domains and the stability of the properly folded forms will be required before these antibodies are tested in vivo. The approaches we intend to explore include the use of different linkers and the engineering of V region residues that optimize the association of homologous $V_H$ and $V_L$ chains.

### *Effect of antigen density and antibody affinity on targeting tumor cells*

Fluorescein can be covalently linked to the surface of the tumor cells through the isothiocyanate derivative FITC. By varying the amount of FITC added to tumor cells, it is possible to obtain target cells with a range of different fluorescein densities. The number of antibody-accessible fluoresceins can be determined by saturation binding with $^{125}$I-labeled antifluorescein. To evaluate the role of antigen density in the targeting with $scFv_2$, the tumor cell lines Daudi and EL4 were labeled at different levels, and the amount of lysis observed with CTL 2C in the presence of a constant concentration of $scFv_2$ antibody was examined (Table 1). Lysis was observed at the highest densities examined (>20,000/cell), but a significant reduction was observed when the densities were lower (<3000/cell).

To examine the role of antibody affinity in targeting tumor cells, we took advantage of the following observations. The fluorescein-specific $scFv_2$ described previously binds to free fluorescein with a $K_d$ of ~1 nM, but it also binds to other fluorescein analogs with a range of different affinities. For example, fluorescein isothiocyanate that is linked through the isomer 5 position of the phenyl ring (FITC-I) has approximately 50 times higher affinity than fluorescein isothiocyanate that is linked through the isomer 6 position of the phenyl ring (FITC-II) (Fig. 3). Fluorescein attached to the lipid phosphatidylethanolamine, Fl-DHPE, has an affinity that is intermediate between these two compounds. Each of these compounds was attached to Daudi or EL4 cells at various densities, and the approximate number of accessible sites was measured. The fluorescein-labeled tumor cells were examined as targets in a cytotoxicity assay with CTL 2C at a constant concentration of the $scFv_2$ antibody (Table 1). Although target cells that bear FITC-I and Fl-DHPE, the two highest affinity ligands, were lysed efficiently, target cells that bear FITC-II, the lowest affinity ligand, failed to be lysed.

The data described can be summarized as shown in Figure 4. It is likely that these curves would shift depending on the concentration of BsAb, the intrinsic susceptibility of the target cell, and the nature of the effec-

TABLE 1. EFFECT OF ANTIGEN DENSITY AND ANTIBODY AFFINITY ON TARGET CELL LYSIS MEDIATED BY scBsAb 1B2/4420

| *Fluorescein label (target cell)* | *Fluorescein/cell (accessible)* | *Lysis ($^{51}$Cr-release)* |
|---|---|---|
| Experiment 1 (Daudi) | | |
| $10^{-4}$ M FITC-I | 380,000 | 64 ± 3% |
| $10^{-5}$ M FITC-I | 24,000 | 48 ± 2% |
| $10^{-6}$ M FITC-I | 2,200 | 2 ± 1% |
| $10^{-7}$ M FITC-I | 500 | 2 ± 1% |
| Experiment 2 (Daudi) | | |
| $10^{-4}$ M FITC-I | 300,000 | 36 ± 2% |
| $10^{-5}$ M FITC-I | 30,000 | 17 ± 2% |
| $10^{-4}$ M FITC-II | 270,000 | 0 ± 1% |
| $10^{-5}$ M FITC-II | 18,000 | 0 ± 1% |
| Experiment 3 (Daudi) | | |
| $10^{-5}$ M Fl-DHPE | 230,000 | 45 ± 4% |
| $10^{-6}$ M Fl-DHPE | 54,000 | 49 ± 2% |
| $10^{-7}$ M Fl-DHPE | 19,000 | 0 ± 1% |
| $10^{-8}$ M Fl-DHPE | 2,000 | 0 ± 1% |
| Experiment 4 (EL4) | | |
| $10^{-4}$ M FITC-I | 340,000 | 20 ± 4% |
| $10^{-5}$ M FITC-I | 19,000 | 6 ± 2% |
| $10^{-6}$ M FITC-I | 2,400 | 3 ± 1% |
| $10^{-7}$ M FITC-I | 700 | 3 ± 2% |
| $10^{-4}$ M FITC-II | 240,000 | 4 ± 2% |
| $10^{-5}$ M FITC-II | 14,000 | 3 ± 1% |
| Experiment 5 (EL4) | | |
| $10^{-5}$ M Fl-DHPE | 340,000 | 35 ± 3% |
| $10^{-6}$ M Fl-DHPE | 93,000 | 35 ± 2% |
| $10^{-7}$ M Fl-DHPE | 9,000 | 8 ± 1% |
| $10^{-8}$ M Fl-DHPE | 600 | 5 ± 1% |

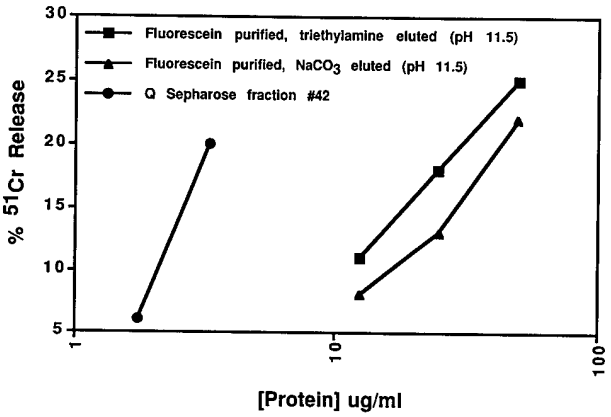


**FIG. 2.** Cytotoxicity assay of various $scFv_2$ preparations. Affinity-purified protein eluted at high pH in either triethylamine or $NaCO_3$ was compared with Q-Sepharose-purified fraction 42 (see Fig. 1). Samples were examined in a 4 h $^{51}$Cr-release assay with CTL 2C in triplicate at the two concentrations shown.

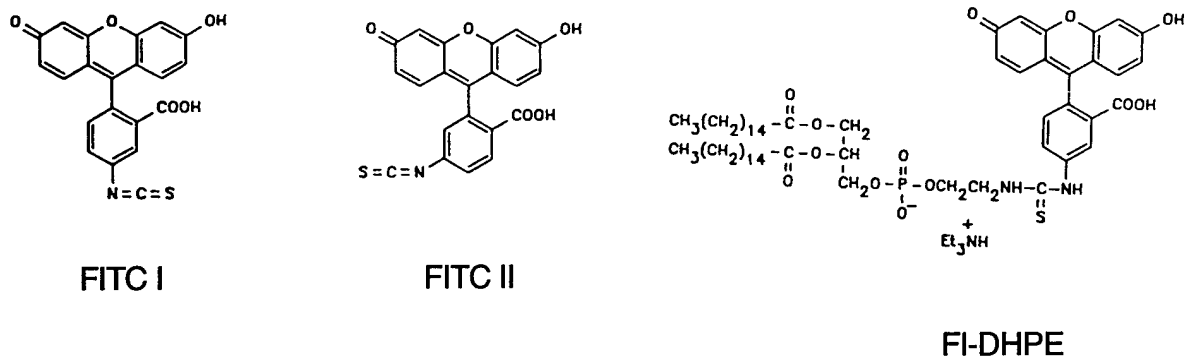


**FIG. 3.** Structures of fluorescein compounds used as surrogate antigens coupled to tumor cells.

tor cell population. Nevertheless, the results show that it should be possible to select, or engineer, antibodies with appropriate affinities (i.e., affinities that will target tumor cells that bear a particular range of antigen densities but will spare normal cells that might have densities at reduced levels).

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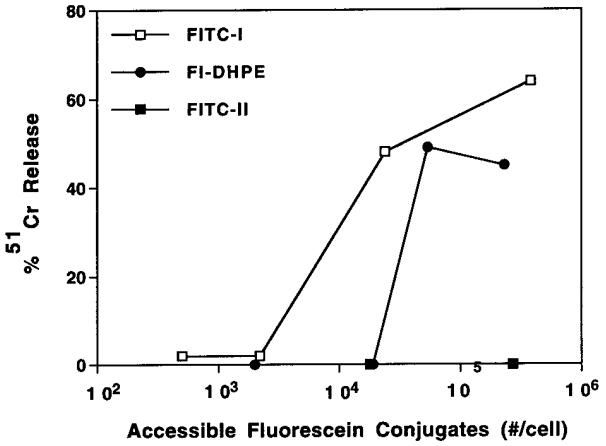


**FIG. 4.** Effect of antigen density and antibody affinity on ability of bispecific $scFv_2$ to mediate lysis of target cells. Daudi tumor cells were $^{51}Cr$-labeled and incubated with various concentrations of FITC-I, Fl-DHPE, or FITC-II (see Materials and Methods). The labeled cells were incubated with CTL 2C and BsAb 1B2/4420 in a 4 h $^{51}Cr$-release assay. The estimated $K_d$s of the $scFv_2$ for the ligands were determined to be 1 nM, 6 nM, and 50 nM (calculated at ambient temperature).

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# Conjugates of folate and anti-T-cell-receptor antibodies specifically target folate-receptor-positive tumor cells for lysis

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**ABSTRACT** **High-affinity folate receptors (FRs) are expressed at elevated levels on many human tumors. Bispecific antibodies that bind the FR and the T-cell receptor (TCR) mediate lysis of these tumor cells by cytotoxic T lymphocytes. In this report, conjugates that consist of folate covalently linked to anti-TCR antibodies are shown to be potent in mediating lysis of tumor cells that express either the $\alpha$ or $\beta$ isoform of the FR. Intact antibodies with an average of five folates per molecule exhibited high affinity for $FR^+$ tumor cells but did not bind to $FR^-$ tumor cells. Lysis of $FR^+$ cell lines could be detected at concentrations as low as 1 pM ($\approx$0.1 ng/ml), which was 1/1000th the concentration required to detect binding to the $FR^+$ cells. Various $FR^+$ mouse tumor cell lines could be targeted with each of three different anti-TCR antibodies that were tested as conjugates. The antibodies included 1B2, a clonotypic antibody specific for the cytotoxic T cell clone 2C; KJ16, an anti-V$\beta$8 antibody; and 2C11, an anti-CD3 antibody. These antibodies differ in affinities by up to 100-fold, yet the cytolytic capabilities of the folate/antibody conjugates differed by no more than 10-fold. The reduced size (in comparison with bispecific antibodies) and high affinity of folate conjugates suggest that they may be useful as immunotherapeutic agents in targeting tumors that express folate receptors.**

High-affinity folate receptors (FRs) with a $K_d \approx 1$ nM have recently been detected on the surface of a number of different types of human cancers, particularly ovarian carcinomas and some types of brain tumors (1–4). These receptors differ from the lower affinity reduced-folate/methotrexate (MTX) carriers ($K_d \approx 1$–100 $\mu$M) that appear to be largely responsible for the transport of folate-based dihydrofolate reductase inhibitors, such as MTX (5, 6). The presence of FR on human tumor cells has led to the use of FR as a target for specific monoclonal antibodies, such as MOv18 and MOv19 (3). Targeting approaches with monoclonal anti-FR antibodies have included the following: $^{131}$I-labeled antibodies (7), engineering of constant regions to optimize antibody-dependent cellular cytotoxicity (8), and bispecific antibodies that target immune effector cells to the $FR^+$ tumor (9–11). The latter studies have used anti-FR antibodies linked to either anti-Fc receptor antibodies or to anti-CD3 antibodies for recruitment of monocytes/natural killer cells or cytotoxic T cells, respectively. Clinical trials with the radiolabeled antibodies and the anti-FR/anti-CD3 bispecific antibodies have recently been initiated (7, 12, 13).

Another approach to targeting $FR^+$ tumor cells has relied on the ability of the FR to endocytose proteins that are covalently linked to folate. For example, Low and colleagues have shown that tumor cells internalize momordin/folate (14) and *Pseudomonas* exotoxin/folate (15) conjugates and liposomes containing chemotherapeutic compounds (16).

Monoclonal antibodies to the mouse FR have not been produced, and, thus, antibody targeting of the FR on mouse tumor cells as a model for human disease has not been possible. Nevertheless, two mouse homologs of the human FR isoforms have been identified, and these receptors also bind folate with high affinity ($K_d \approx 1$ nM) (17, 18). Several mouse leukemia lines have been selected for high FR expression by growth in medium with low concentrations of folate. Two forms ($\alpha$ and $\beta$) of the mouse FR have been identified as 38-kDa, lipid-linked membrane glycoproteins (19). As in humans, FRs also appear to be expressed at high levels on some mouse tumors. For example, mouse choroid plexus tumors that arise in mice transgenic for the simian virus 40 large T antigen (20) express high levels of FR (E.J.R., T.A.P., D.M.K., and T. Van Dyke, unpublished data).

A previous study showed that conjugates of a peptide analog of melanocyte-stimulating hormone and an anti-CD3 antibody mediated lysis of melanoma cells by cytotoxic T lymphocytes (CTLs) (21). The high affinity of FR for folate suggested that attachment of folate directly to an anti-T-cell receptor (TCR) antibody might likewise efficiently target $FR^+$ tumor cells. We show in this report that these conjugates can mediate lysis of the mouse $FR^+$ tumor cells at very low concentrations ($\approx$1 pM). In addition, tumor cell lines with a range of FR densities could be killed, while the parental line with no detectable FR was spared. The effectiveness is likely to be due in part to the finding that the binding affinity of the folate-linked antibody is nearly as high as free folate for the FR $\alpha$ and $\beta$ forms. This property, together with previous observations that only a few TCRs may need to be engaged to activate a CTL (22), probably accounts for the potency of the folate/anti-TCR conjugates as tumor-targeting agents. Furthermore, the smaller size of the folate/anti-TCR antibody conjugate compared with bispecific antibodies that contain an anti-TCR antibody coupled to an anti-FR antibody may have some therapeutic advantage.

## MATERIALS AND METHODS

**Cell Lines and Monoclonal Antibodies.** The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 medium containing 5 mM Hepes, 10% (vol/vol) fetal bovine serum, 1.3 mM L-glutamine, 50 $\mu$M 2-mercaptoethanol, 100 units of penicillin per ml, and 100 $\mu$g of streptomycin per ml: Mel, murine erythroleukemia cell (23); La, a subline of Mel selected on low folate (19); L1210, a murine leukemia cell line (24); LL3, a subline of L1210 selected on low 5-formyltetrahydrofolate (17); and F2-MTX$^r$A, a MTX-resistant L1210 subline selected for increased expression of FR$\beta$ by growth on low folic acid (25). La and LL3 express the $\alpha$ form of the folate receptor, and F2-MTX$^r$A expresses the $\beta$ form of the folate receptor. CTL clone 2C, a mouse alloreactive cell line specific for $L^d$, was maintained in the same RPMI medium

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Abbreviations: FR, folate receptor; CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; MTX, methotrexate; MHC, major histocompatibility complex; SEB, staphylococcal enterotoxin B.

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described above and supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5% α-methyl mannoside, and mitomycin C-treated BALB/c spleen cells as stimulators (26). Monoclonal antibody 1B2, a mouse IgG1 specific for the TCR of CTL 2C, was purified from ascites (27). Hybridoma KJ16, a rat IgG antibody specific for the Vβ8 region of the TCR (28), was cultured in low-serum medium (1% fetal bovine serum in Dulbecco's modified Eagle's medium) in a bioreactor (Amicon), and the antibody was concentrated by ammonium sulfate precipitation. Hybridoma 2C11, a hamster IgG specific for the mouse CD3 ε subunit (29), was cultured in serum-free medium (GIBCO/BRL) and purified over a protein G-Sepharose column. Hybridomas that secrete antibodies to major histocompatibility complex (MHC) class I $L^d$, 30.5.7 (30), and $K^dD^d$, 34.1.2s (31), were cultured in the RPMI medium described above and used in flow cytometry without further purification. 30.5.7 was also prepared as ascites fluid and used without further purification in some cytotoxicity assays. Fluorescein-labeled, anti-IgG antibodies were obtained from Kirkegaard & Perry Laboratories.

Polyclonal BALB/c effector cells enriched for activated CTLs were obtained by incubation of $5 \times 10^6$ spleen cells per ml with 10 μg of staphylococcal enterotoxin B (SEB) per ml (Toxin Technologies, Madison, WI), 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, and 5% α-methyl mannoside (32). Cells were used 3 or 4 days after stimulation.

**Preparation of Folate/Antibody Conjugates.** Folate was coupled through carboxyl groups to antibody amine groups by using a carbodiimide procedure (14). A 5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 min at room temperature in the dark, a 10- or 100-fold molar excess of the EDC-activated folate was added to 0.5–2.0 mg of antibody in 0.1 M Mops, pH 7.5. After 1 h at room temperature, the sample was applied to a Sephadex G-25 column equilibrated in phosphate-buffered saline (PBS; 10 mM sodium phosphate/150 mM sodium chloride, pH 7.0). The excluded-peak fractions were pooled and analyzed spectrophotometrically at 280 and 363 nm. Epitope densities of the folate on antibody conjugates were determined by using molar extinction coefficients ($\varepsilon_M$) for folate of 6,197 (363 nm) and 25,820 (280 nm). Antibody concentrations were determined by subtracting the absorbance contribution of folate at 280 nm and by using an antibody $\varepsilon_M$ of 224,000. Conjugates were stored at 4°C in the dark.

**Mass Spectrometry.** Mass spectra were obtained on a TofSpec mass spectrometer by using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and concentrated to 10–25 pmol/ml. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois.

**Folate-Binding Assays.** Binding assays were conducted by using $^{125}$I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.4 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 75 μl of PBS-BSA for 1 h. Bound and free ligand were separated by centrifugation through oil [80% (vol/vol) dibutyl phthalate/20% (vol/vol) olive oil] at $12,000 \times g$ for 3 sec. Tubes were frozen and cut to allow the radioactivity in the pelleted cells and supernatants to be quantitated separately. Binding parameters were calculated by using LIGAND (33).

**Cytotoxicity Assays.** Tumor cells were labeled with 50 μl of $^{51}$Cr (2.5 mCi/ml) for 60 min at 37°C, washed twice with folate-free RPMI 1640 medium containing 5% (vol/vol) fetal calf serum (folate-free media), and used in 96-well plate cytoxicity assays at $10^4$ cells per well. Antibodies and folate/antibody conjugates were added to triplicate wells at various concentrations diluted in folate-free medium. For folate-inhibition studies, folate was added to a final concentration of 2.5 μM. Effector cells (2C or SEB-reactive polyclonal BALB/c T cells) were added at effector-to-target cell ratios of 5:1 or 10:1. For experiments with 2C as the effector cells, the anti-$L^d$ antibody was used at a 1:100 dilution of ascites to inhibit recognition of the $L^d$ alloantigen by CTL 2C. Plates were incubated at 37°C for 4 h, and supernatants were removed for γ counting. Specific $^{51}$Cr release was calculated by standard methods. Where indicated the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates.

## RESULTS

**Characterization of Folate/Anti-TCR Antibody Conjugates.** The anti-clonotypic antibody 1B2 has a high intrinsic affinity ($K_d \approx 1$ nM) for the TCR on the mouse CTL clone 2C (34). To explore the potential of folate/anti-TCR conjugates, 1B2 was coupled at molar ratios of folate to antibody of 10:1 and 100:1. The 10:1 and 100:1 ratios yielded preparations containing an average molar ratio of 1.3 folates per antibody and 6.0 folates per antibody, respectively.

To confirm these values and to determine the range of epitope densities among the antibodies within a single preparation, the folate/1B2 conjugate (100:1) was examined by electrospray ionization mass spectrometry (Fig. 1). The conjugate had an average molecular mass of 148,935, while the unlabeled 1B2 exhibited a mass of 147,140. By this estimate, the preparation contained an average of 4.1 folates per antibody. Analysis of separate heavy and light chain profiles yielded a value of 4.9 folates per antibody (data not shown). Integration of the mass spectrometry peak indicated that >95% of the antibody molecules contained fewer than 10 folate molecules.

Two additional anti-TCR antibodies, KJ16 and 2C11, were also coupled with folate at the 100:1 molar ratio of folate to antibody. These antibodies exhibit different affinities from 1B2, and they recognize TCR epitopes on the Vβ region and CD3 molecules present on mouse CTL 2C (refs. 28, 29, and 35; Yuri Sykulev and Herman N. Eisen, personal communication). Thus, analysis of these folate/antibody conjugates would allow us to determine if either TCR epitope or antibody affinity affected tumor cell targeting. Each of these preparations exhibited folate densities that were similar to 1B2 (4.9 for KJ16 and 4.4 for 2C11, as judged by spectrophotometry).

**Binding of Conjugates to Tumor Cell Lines.** To determine if the folate/antibody conjugates bind to the FR on the surface of tumor cells, a competition assay with $^{125}$I-labeled folate as the labeled ligand was used. Because cytotoxicity assays are performed at 37°C, all binding studies shown here were also done at 37°C. Previous reports have measured folate binding to FR at 4°C and used a low pH wash to remove endogenously bound folate (19, 36). We determined that conducting the assay at 37°C would allow exchange of labeled and unlabeled

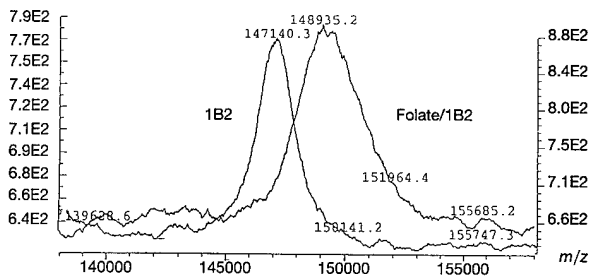


FIG. 1. Mass spectra of anti-TCR antibody 1B2 and folate/1B2 conjugate. The shift in molecular mass of the major peak indicates that an average of approximately four folates are attached to intact 1B2 IgG1.

ligands and produce similar levels of binding compared with acid pretreatment and a 0°C incubation (data not shown). We also determined the affinity of $^{125}$I-labeled folate for the FR$\alpha$ and FR$\beta$ isoforms of the receptor at 0°C and 37°C by using the FR$\alpha^+$ and FR$\beta^+$ lines La and F2-MTX$^r$A (Table 1 and Fig. 2 *A* and *B*).

A competition experiment was performed with both cell lines with folate/1B2 (100:1) (Fig. 2 *A* and *B*), folate/1B2 (10:1) (data not shown), and unlabeled 1B2 (data not shown). Both conjugates but not the unlabeled 1B2 inhibited the binding of the labeled ligand. Folate in the conjugated form was about 1/10th as effective at binding than was free folate. This is probably due in part to the fact that carbodiimide coupling can occur through either the $\alpha$ or the $\gamma$ carboxyl of folate, and only the latter retains binding to FR (15). In addition, it is possible that linkage to some amino groups on the antibody may result in steric hindrance to the FR. Nevertheless, the average $K_d$ of the folate/1B2 preparation for the two cell lines was determined to be 20 nM and 60 nM for FR$\alpha$ and FR$\beta$, respectively.

A comparison of the three different folate/anti-TCR antibody conjugates is shown in Fig. 2*C*. All three conjugates were capable of inhibiting the binding of $^{125}$I-labeled folate to FR$^+$ cells. Inhibition was not observed with unconjugated antibodies. The calculated $K_d$ values of the 1B2, KJ16, and 2C11 conjugates in this experiment were 80 nM, 90 nM, and 50 nM, respectively. These similarities indicate that any significant differences in the targeting effectiveness of these antibodies (shown below) are likely to depend on factors other than their folate density.

**Conjugates Mediate Specific Lysis of Tumor Cells by a Mouse CTL Clone.** To test the targeting efficiency and specificity of the folate conjugates, five different mouse tumor cell lines were examined in a $^{51}$Cr-release assay with the mouse CTL clone 2C. Each of these lines was also examined for binding of $^{125}$I-labeled folate to approximate the number of FRs at 37°C (Table 1). As expected, the highest levels of expression were detected for the low-folate-selected lines F2-MTX$^r$A, La, and LL3 (19). The parental line L1210 had a low but detectable level of FR, and the parental line Mel had no detectable FR.

Because each of these lines also express the alloantigen L$^d$ that is recognized by CTL clone 2C, assays were performed in the presence of excess monoclonal anti-L$^d$ antibody to minimize non-FR-mediated lysis. As shown in Fig. 3, lysis of each of the FR$^+$ cell lines was detected in the presence of the folate/1B2 conjugate. The lysis was completely inhibited by free folate, indicating that it was mediated by binding to the FR and not by other cell surface molecules—e.g., Fc receptors.

Table 1. Characteristics of cells used as targets for lysis

| Cell line | FR type | $B_{max}$, sites/cell* | $K_d$, nM 0° | $K_d$, nM 37° | Class I, mean fluorescent units† L$^d$ | Class I, mean fluorescent units† K$^d$D$^d$ | Class I, mean fluorescent units† No Ab |
|---|---|---|---|---|---|---|---|
| F2-MTX$^r$A | $\beta$ | 200,000 | 0.7 | 5 | 131 | 174 | 4 |
| La | $\alpha$ | 60,000 | 0.9 | 1 | 64 | 76 | 4 |
| LL3 | $\alpha$ | 20,000 | 0.3 | ND | 144 | 183 | 5 |
| L1210 | — | 8,000‡ | ND | ND | 125 | 154 | 3 |
| Mel | — | <4,000‡ | ND | ND | 61 | 79 | 4 |

Ab, antibody; ND, not determined.

*Except where indicated, $B_{max}$ was determined from nonlinear regression from the competition curve by using $^{125}$I-labeled folate and unlabeled folate.

†The levels of MHC class I surface antigens were examined by flow cytometry, as described in ref. 32.

‡Values were estimated from a single binding experiment with $^{125}$I-labeled folate at 0.7 nM, in comparison with LL3. At 0.7 nM $^{125}$I-labeled folate, specific cpm bound per $10^6$ cells were 32,000 (F2-MTX$^r$A), 43,000 (La), 15,000 (LL3), 600 (L1210), and <300 (Mel).

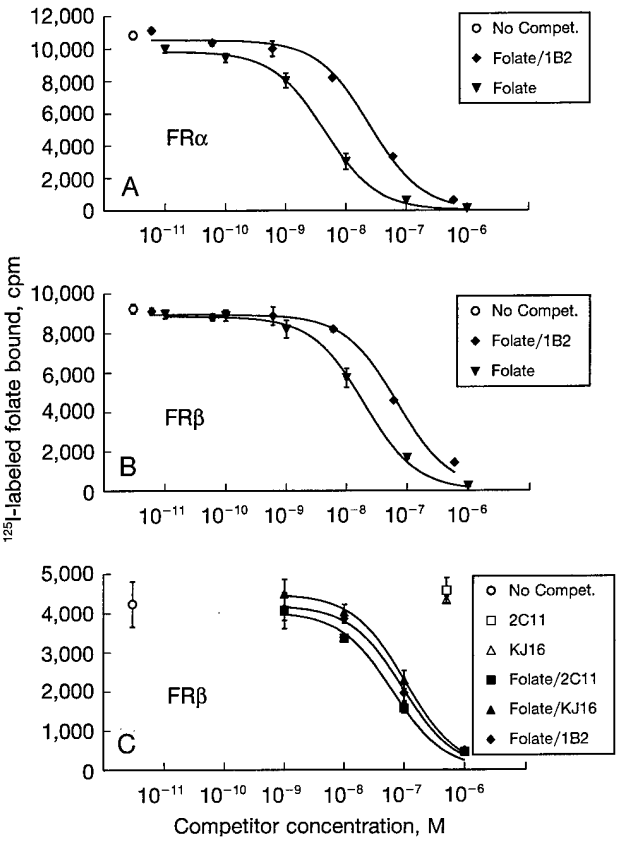


FIG. 2. Inhibition-binding curves of free folate and folate/antibody conjugates. $^{125}$I-labeled folate was incubated with two tumor cell types in the presence or absence of competitors for 1 h at 37°C. Concentrations refer to folate rather than antibody concentrations, except in the case of *C*, where unconjugated antibodies were also tested for competition. (*A*) La cells, which express FR$\alpha$. (*B*) F2-MTX$^r$A cells, which express FR$\beta$. (*C*) Conjugates of folate and three anti-T-cell antibodies were compared in their ability to compete with $^{125}$I-labeled folate for binding to F2-MTX$^r$A cells (FR$\beta$). The unconjugated antibodies 2C11 and KJ16 showed no inhibition of $^{125}$I-labeled folate binding, as did 1B2 in a separate experiment (data not shown).

The extent of lysis was correlated with the level of surface FR, and the F2-MTX$^r$A line always exhibited more sensitivity than the other lines. In contrast, the FR$^-$ cell line Mel was not lysed, even at a folate/1B2 concentration that was 1000 times higher than the concentration required for detectable killing of the FR$^+$ line F2-MTX$^r$A.

To determine if the three different folate/anti-TCR antibody conjugates were all effective at mediating specific lysis, the cell line with the highest FR level, F2-MTX$^r$A (Fig. 4*A*) and the one with lowest—i.e., undetectable—FR level, Mel (Fig. 4*B*) were assayed at various conjugate concentrations. Each of the conjugates mediated lysis of F2-MTX$^r$A but not Mel. The lysis was specific, as indicated by the ability of free folate to inhibit lysis by each of the conjugates.

**Conjugates Mediate Specific Lysis of Tumor Cells by Polyclonal CTLs.** Activated T cells from a BALB/c mouse were obtained by *in vitro* stimulation of spleen cells with SEB (32). This T-cell population is enriched for V$\beta$8$^+$/KJ16-reactive cells, although they do not express the epitope of the clonotypic antibody 1B2. The FR$^+$ F2-MTX$^r$A line was efficiently lysed by the BALB/c-derived T cells in the presence of the folate/KJ16 and folate/2C11 conjugates. As expected, lysis was not observed with the folate/1B2 conjugate (Fig. 5*A*). In a separate experiment with polyclonal CTLs, folate/KJ16 and folate/2C11 mediated lysis was shown to be completely inhibited by free folate (data not shown). In contrast, there was

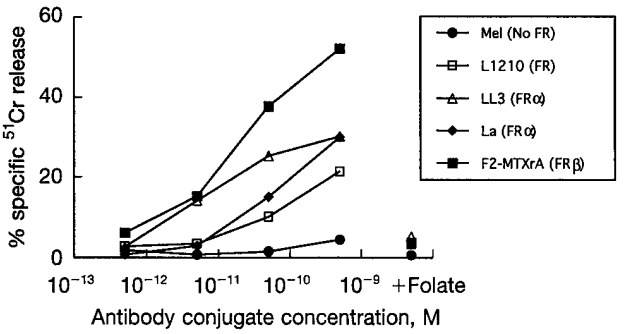


FIG. 3. Cytotoxicity assay of various tumor cell lines with folate/anti-TCR antibody 1B2 and CTL clone 2C. Specific $^{51}$Cr release mediated by the indicated concentration of the folate/1B2 conjugate (conjugate with approximately four folates per antibody) was determined in the presence of the anti-$L^d$ antibody to minimize lysis due to recognition of $L^d$, the nominal ligand for CTL 2C. The $^{51}$Cr release measured with 2C and anti-$L^d$ antibody but in the absence of conjugate (0% for Mel, 3% for F2-MTX$^r$A, 0% for La, 37% for L1210, 5% for LL3) was subtracted to yield the values shown. The background values of $^{51}$Cr release may have varied between different target cells depending on the level of surface $L^d$ (see Table 1). Assays were incubated for 4 h at an effector-to-target cell ratio of 5:1. Assays with free folate at a final concentration of 2.5 $\mu$M were performed with the second highest concentration (0.05 nM) of the folate/1B2 conjugate.

relatively little effect on the FR$^-$ line Mel, although at the highest concentration of 2C11, there was some lysis (Fig. 5*B*). This could be due to antibody-dependent cellular cytotoxicity mediated by the conjugate and non-T-cell effectors within the polyclonal population. Alternatively, there may be low levels of FR that are not detectable by standard binding assays.

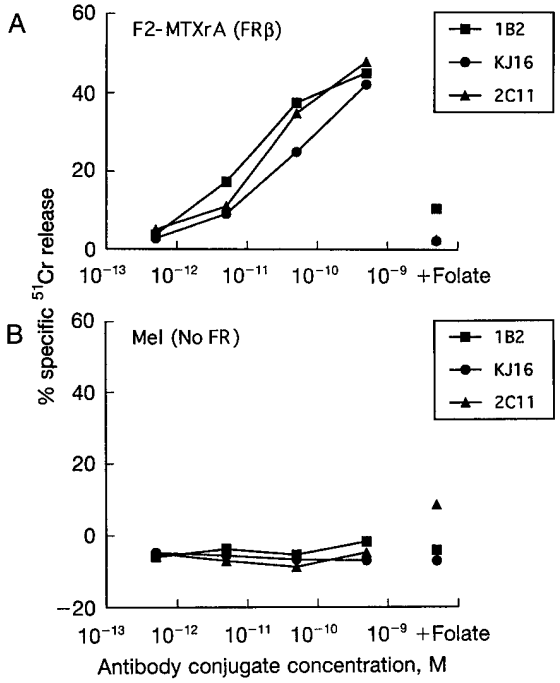


FIG. 4. Cytotoxicity assay of FR$^+$ and FR$^-$ tumor cell lines with three different folate/anti-TCR conjugates and CTL clone 2C. Assays were performed as described in the legend to Fig. 4 at an effector-to-target cell ratio of 10:1. Assays with free folate at a final concentration of 2.5 $\mu$M were performed with the highest concentration (0.5 nM) of the folate/1B2 conjugate. (*A*) Lysis of the FR$^+$ cell line F2-MTX$^r$A. $^{51}$Cr release with 2C and anti-$L^d$ antibody but in the absence of folate conjugates was 0%. (*B*) Lysis of the FR$^-$ cell line Mel. $^{51}$Cr release with 2C and anti-$L^d$ antibody but in the absence of folate conjugates was 0%.

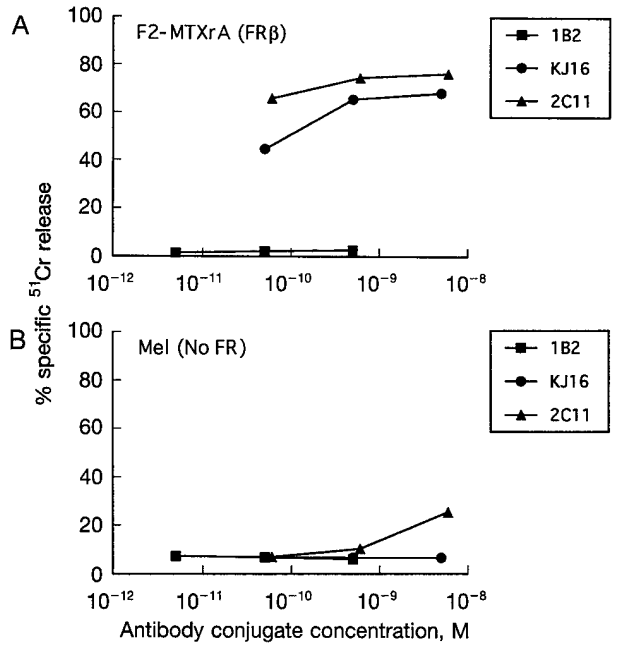


FIG. 5. Cytotoxicity assay of FR$^+$ and FR$^-$ tumor cell lines with three different folate/anti-TCR conjugates and polyclonal BALB/c T cells. SEB-stimulated spleen cells were used as a source of effector cells at an effector-to-target cell ratio of 10:1. (*A*) Lysis of the FR$^+$ cell line F2-MTX$^r$A. $^{51}$Cr release with effector cells but in the absence of folate conjugates (27%) was subtracted to yield the indicated values. (*B*) Lysis of the FR$^-$ cell line Mel. $^{51}$Cr release with effector cells but in the absence of folate conjugates (17%) was subtracted to yield the indicated values.

## DISCUSSION

Bispecific antibodies that mediate lysis of tumor cells by activated T cells, natural killer cells, monocytes, or macrophages have been shown to be effective in various animal models of disease (reviewed in ref. 37). Monoclonal antibodies to the human FR linked to an anti-CD3 antibody are able to retarget the lysis of these tumor cells *in vitro*. A clinical trial that targets the high-affinity FR present on ovarian tumors with anti-FR × anti-CD3 bispecific antibodies has recently been initiated (7).

In this report, we describe an alternative approach to targeting FR$^+$ tumors for immune-mediated lysis. This approach may offer several advantages over conventional bispecific antibodies. The method takes advantage of the high affinity of folic acid for the FR ($K_d \approx 1$ nM) compared with the affinity of the constitutive reduced folate carrier protein ($K_d \approx 100$ $\mu$M) that is expressed by most cells (6). Because the affinity of folate for the FR is of the same order as the highest affinity antibodies, we reasoned that a simple conjugate of folate and the anti-TCR antibody may be as effective as the best bispecific antibodies. Indeed, the folate/anti-TCR conjugates bind to the FR with approximately 1/10th of the affinity compared with free folate, and they mediate specific lysis of the FR$^+$ tumor cells.

Two observations regarding the effectiveness of the folate-targeting approach suggest that it should be generally useful. First, all three different anti-TCR antibodies mediated lysis with less than a ten-fold difference between them, despite the fact that these antibodies differ in affinity by at least 100-fold: 1B2, $K_d \approx 1$ nM (34); KJ16, $K_d \approx 100$ nM (35); 2C11, $K_d > 10$ nM (Yuri Sykulev and Herman N. Eisen, personal communication). Second, four different tumor cell lines with a wide range of densities of the high-affinity FR were specifically lysed while the FR$^-$ tumor line was spared. The latter result

indicates that the ubiquitous expression of folate carrier protein by cells may not result in destruction of most normal cells.

We have noticed that tumor lines with relatively high levels of the FR are not lysed equally well—e.g., F2-MTX$^r$A vs. La; Fig. 2. Clearly there are other factors that contribute to efficient recognition and lysis mediated by the folate/antibody conjugates, just as there are with conventional bispecific antibodies (reviewed in ref. 37). These factors include adhesion molecule levels and intrinsic susceptibilities of the tumor cells. With the F2-MTX$^r$A line, it may be related to the level of MHC class I protein present on this cell compared with the amount on La (Table 1).

It is likely that the folate conjugates described here can be optimized further by engineering antibodies for uniform coupling of folate through the γ-carboxyl. For example, a 10-fold increase in folate/toxin effectiveness was observed when only the γ-carboxyl of folate was coupled through a disulfide bond rather than coupling through carbodiimide-mediated linkages (15). We have previously described the cloning of a single-chain 1B2 antibody (34) and have recently prepared an active single-chain version of KJ16 (Bryan Cho and D.M.K., unpublished data). Jost *et al.* (38) have also described an active anti-CD3 single-chain antibody. Thus, it should soon be possible to create folate/single-chain Fv conjugates and to test their effectiveness in comparison with the intact antibodies described in this report.

The *in vivo* effectiveness of tumor-targeting agents may be influenced by additional factors, such as interactions with serum components, other immune effectors, and normal tissues, but several advantages of the conjugates described here are worth noting. The reduced size and immunogenicity of folate/antibody conjugates should provide some advantage over conventional bispecific antibodies. Folate/antibody conjugates are approximately one-half the size of bispecific antibodies. Thus, folate/single-chain Fv regions will be approximately 30 kDa compared with 60 kDa for the bispecific antibodies which is currently the smallest active form of a bispecific antibody—i.e., two linked Fv regions (39). This reduced size should result in improved tumor penetration and tumor/tissue localization ratios (40). Finally, immunogenicity may be reduced since human anti-immunoglobulin responses to the anti-FR cannot occur when folate is used directly as the targeting moiety.

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*Laboratory Investigation*

# Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice

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*Key words:* brain tumor, choroid plexus carcinoma, ependymoma, SV40 T antigen, folate binding protein, SV11 mice

**Summary**

A high affinity folate receptor is expressed in some human cancers, including choroid plexus tumors and ependymomas, and has been suggested as a target for therapeutics. In this report, the expression of folate receptors in an SV40 large T antigen transgenic mouse (SV11) was investigated. SV11 mice develop choroid plexus tumors, a property that may be related to the observation that SV40 has been isolated from human choroid plexus tumors and ependymomas. We report that SV11 choroid plexus tumors contain a high affinity folate receptor ($K_D$ of 1 nM), detectable by $^{125}$I-folate autoradiography and immunohistochemistry. Western blot analysis indicated an apparent molecular weight of 38 kDa. RT-PCR revealed the presence of transcripts for both alpha and beta isoforms of the folate receptor. Brain parenchyma has undetectable folate receptor, but normal choroid plexus has substantial levels (as does human choroid plexus). The folate receptors of the tumor are accessible from the bloodstream whereas those of the normal choroid plexus are not. Thus SV11 transgenic mice should be useful for evaluating therapeutic targeting of high affinity folate receptors, both for efficacy of specific agents and possible side effects.

## Introduction

The choroid plexus is a specialized region of ependyma, the epithelial lining of the brain ventricles. The incidence of choroid plexus tumors and ependymomas is disproportionately high in young children: 40–70% of each type of tumor is diagnosed in children under two years of age [1, 2]. Pediatric ependymomas and choroid plexus tumors may have an unusual viral etiology. The genomic sequence of the SV40 viral large T antigen (Tag) was detected by PCR in nearly all pediatric ependymomas and in half of choroid plexus tumors examined [3]. The large T antigen was detectable by immunohistochemistry in these tumors. In contrast, other tumor types did not have detectable viral DNA or immunoreactivity. Furthermore, the SV40 virus has subsequently been recovered from the same tumor samples [4].

Mice that are transgenic with SV40 develop ependymal and choroid plexus tumors [5, 6]. One particular line, SV11, develops tumors exclusively in the choroid plexus, and the mice become moribund at $104 \pm 12$ days [7]. The mice are transgenic for the early control region and early genes of the SV40 genome encoding the large T antigen (Tag). Tag is a nuclear protein capable of binding both p53 and pRB; Tag binding of pRB is necessary for Tag-induced tumorigenesis [8]. Tag is expressed in some choroid plexus cells soon after birth and in most

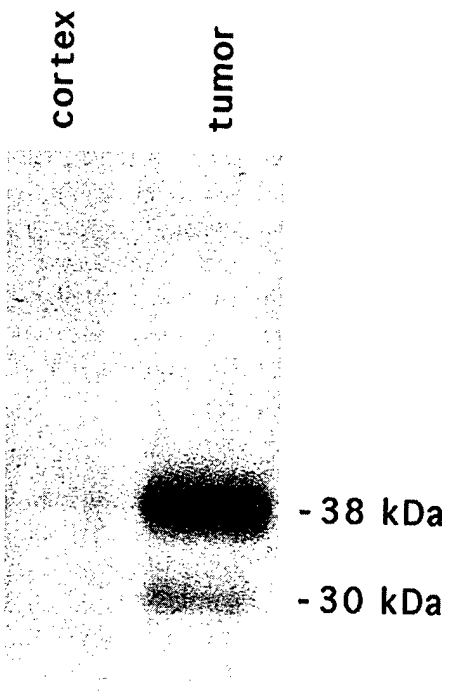



*Figure 1.* Western blot analysis of membrane proteins. Samples of choroid plexus tumor and surrounding brain cortex were homogenized and membranes isolated by differential centrifugation; proteins were detergent solubilized and electrophoresed through 10% polyacrylamide gels (25 µg of tumor protein and 40 µg of cortex protein). After transfer to nitrocellulose membranes, proteins were detected with a polyclonal antiserum against the mouse folate receptor.

*Northern blot analysis*

Forty µg total RNA was denatured in 0.5 X formaldehyde gel-running buffer (1X : 0.02 M 3-(N-morpholino)propanesulfonic acid pH 7.0, 8 mM sodium acetate, 1 mM EDTA), 2.2 M formaldehyde, and 50% (v/v) deionized formamide and the sample was electrophoresed through a 1.5% agarose gel containing 0.66 M formaldehyde and 1 X formaldehyde gel-running buffer. Molecular weight marker lanes were stained separately in 5 µg/ml ethidium bromide, 0.1 M ammonium acetate for 15 minutes and destained in 0.1 M ammonium acetate for two hrs. RNA was transferred to nylon membranes by capillary action in 20 X SSC (1 X SSC: 0.15 M NaCl, 0.015 M $Na_3$ citrate) for a minimum of 16 hrs. Membranes were washed with 2 X SSC and dried prior to baking at 80° C for 2 hrs.

Probes for FR-α and FR-β were made from isolated cDNAs of each (provided by Kevin Brigle). DNA was labeled using a random primer kit (Gibco BRL, Gaithersburg, MD) and [α-$^{32}$P]-dATP (ICN, Costa Mesa, CA) according to the manufacturer's instructions. Membranes were pre-hybridized for 1 hr at 65° C in 0.52 M sodium phosphate (pH 7.2), 15 mM EDTA, 0.25 M NaCl, 6.4% SDS, and 0.01% BSA with 4 µl/ml denatured sonicated salmon sperm DNA. 20 µl heat-denatured [α-$^{32}$P]-labeled probe and an additional 4 µg/ml denatured sonicated salmon sperm were added to the hybridization buffer and allowed to hybridize for a minimum of 12 hrs at 65° C. Membranes were washed successively in 2 X SSC, 0.1% SDS at room temperature, 2 X SSC, 0.1% SDS at 65° C, and 0.2 X SSC, 0.1% SDS at 65° C. The sizes of the major transcripts were calculated by a linear regression plot derived from the marker lane (0.16–1.77 Kb RNA Ladder, Gibco BRL).

*PCR of FR-α and FR-β cDNA*

Plasmid containing cDNAs for FR-α or FR-β was serially diluted by ten-fold increments (5 ng/µl – 500 fg/µl). 1 µl aliquots of each dilution were added to 100 µl of 1 X PCR buffer (50 mM KCl, 10 mM Tris pH 8.4, 1.5 mM $MgCl_2$, 50 mM dNTPs) containing primer pairs (200 nM each) specific to either FR-α or FR-β and 1 unit of Taq polymerase (Fisher). Primer pairs for the FRs amplified the entire coding regions (765 bp for FR-α and 753 bp for FR-β) of each respective cDNA. Each respective set of FR primer pairs flank introns such that amplification of genomic DNA is readily distinguished from cDNA. Sequences of primer pairs were as follows:

FR-α5′ ATGGCTCACCTGATGACTGTGCAG
FR-α3′ GCTGATCACCCAGAGCAGCACTAA
FR-β5′ ATGGCCTGGAAACAGACACCACTC
FR-β3′ GCCAGGGAGCCATAATGACAGCAC

PCR was carried out a total of 35 cycles with a denaturing temperature of 94° C and elongation tem-

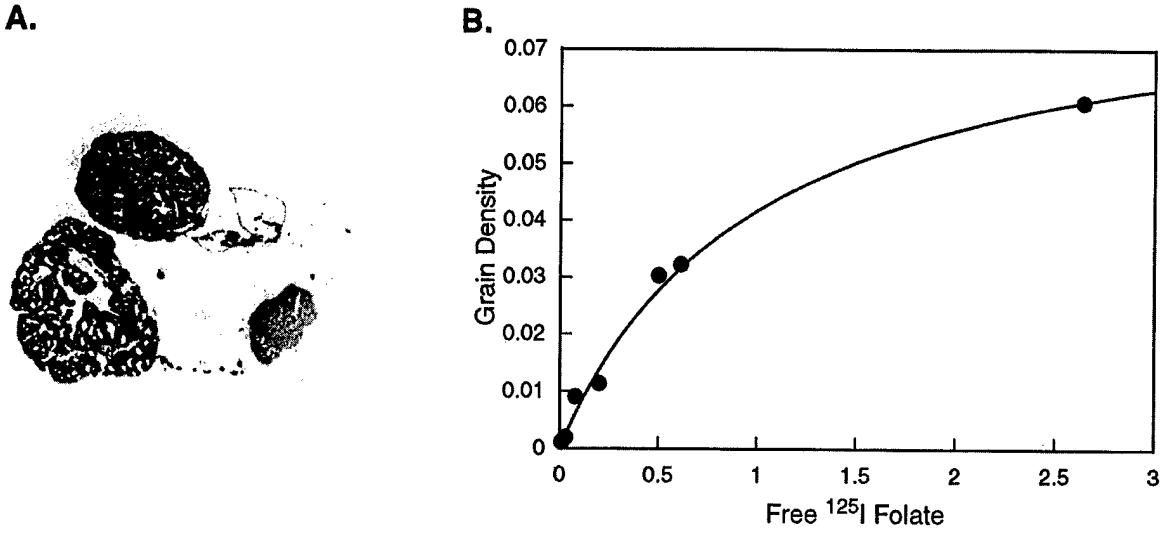



*Figure 2.* Autoradiographic detection of folate receptor binding using $^{125}$I-folate on tissue sections. Cryostat sections of unfixed brain and tumor were incubated with varying concentrations of labeled folate (0.01 nM to 2.6 nM) for 30 min. Slides were dipped in photographic emulsion and exposed for 24 hrs. Sections were counterstained with hematoxylin. *A*. Labeling at 2.6 nM $^{125}$I-folate. *B*. For each concentration of labeled folate, silver grains were counted using the computer program NIH Image. Nonlinear regression analysis of the binding isotherm yielded a $K_D$ of 1 nM.

perature of 72° C for each set of primers. Annealing temperature for FR primer pairs was 57° C.

*RT-PCR*

5 µg of total RNA isolated from cell lines or tumor was heat denatured and added to 1 X cDNA reaction buffer (50 mM KCl, 10 mM Tris pH 8.4, 5 mM DTT, 2.5 mM $MgCl_2$, 0.5 mM dNTPs) with 0.3 µl RNAsin (Promega, Madison, WI), 5 mM oligo-dT, and 45 units AMV reverse transcriptase (Seikagaku, Japan) in a total reaction volume of 20 µl. Reverse transcription reactions were carried out at 42° C for 1 hr. 1 µl of the resulting cDNA reaction mixture was aliquoted into PCR buffer containing either FR-α or FR-β primers and amplified as described.

For RT-PCR analysis of FR isoform expression in tumor and choroid, 0.5 µg of total RNA isolated from normal choroid plexus or tumor was examined as described above to evaluate relative FR isoform expression between tissue samples. Normal choroid plexus was dissected from C57BL/J6 mice 2 hrs after intravenous injection of 0.1 ml 5% (w/v) Evan's Blue dye to facilitate visualization of the choroid plexus. Two-fold dilutions of cDNA reaction mixtures (ranging from 2 µl – 1/256 µl) were used in PCR. β-actin primers, amplifying a 193 bp fragment of β-actin, served as a control for the level of intact mRNA in different samples. Similar to FR primers, β-actin primers flanked at least one intron. Sequences of β-actin primers were as follows:

β-actin 5′ TGCTCTAGACTTCGAGCAGGAG
β-actin 3′ CATGATGGAATTGAATGTAGTT

*Southern blot analysis of RT-PCR products*

Ten µl of each PCR reaction mixture was electrophoresed on a 1.5% agarose gel containing 5 µg/l ethidium bromide. Gels were washed in 0.2 M HCl for 30 min followed by 0.2 M NaOH, 0.6 M NaCl for 30 min, and finally 0.5 M Tris pH 7.5, 1.5 M NaCl for 30 min. DNA was transferred by capillary action to Genescreen nylon membranes in 20 X SSC for a minimum of 12 hrs. Membranes were pre-treated

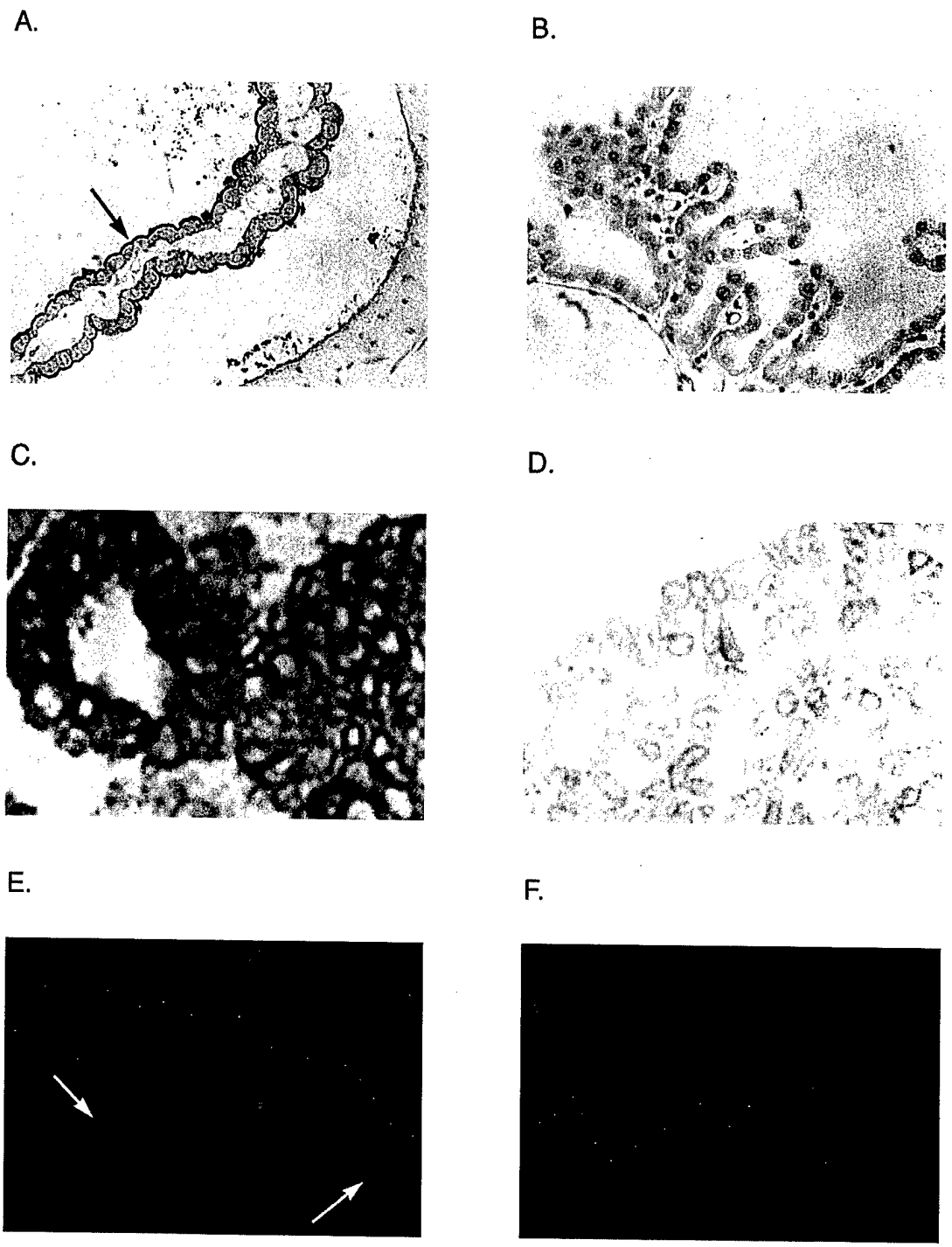



*Figure 3.* Immunohistochemistry of folate receptor in normal choroid plexus and choroid plexus tumor (A–D). A polyclonal rabbit antibody against the mouse folate receptor was used on AZF-fixed, paraffin-embedded tissue sections with the ABC method and metal-enhanced DAB as chromagen. A. Normal choroid plexus (1 : 5,000 dilution of primary antibody). Arrow indicates strong reactivity of apical surface of choroid plexus. B. Normal choroid plexus processed as in A. but with pimary antibody omitted as a control for nonspecific binding. C. Choroid plexus tumor (1 : 5,000 dilution of primary antibody). D. Tumor section with primary antibody omitted. E. Evans Blue dye analysis of accessibility of tissue by blood-borne agent. Staining is primarily in stromal side of normal choroid plexus. Arrows indicate apical surface of choroid plexus and normal brain parenchyma, which are unstained. F. Choroid plexus tumor of same mouse as in E., showing intense staining throughout tumor.

and hybridized with FR-specific probes as described above. Probe for β-actin was isolated from the product of an RT-PCR reaction on murine erythroleukemia cells (Mel). Membranes were washed as described above. Membranes were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) and visualized on a Phosphorlmager (Molecular Dynamics).

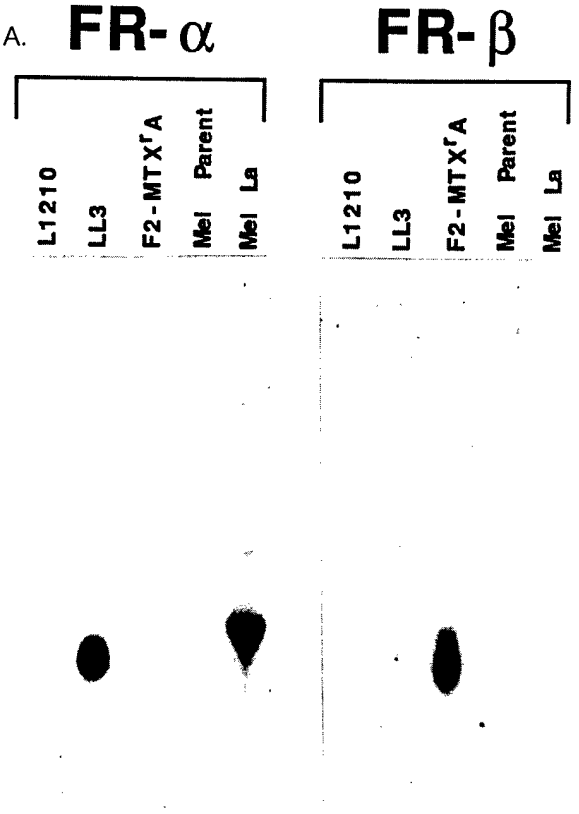
*Blood-Cerebrospinal Fluid (CSF) barrier assessment*

Evans Blue is a vital dye commonly used to assess the intactness of the blood brain barrier [32]. To compare the access of blood-borne substances to tumor versus components of normal choroid plexus, 100 µl of 5% (w/v) Evans Blue (Sigma Chemical, St. Louis, MO) in 10 mM sodium phosphate 150 mM NaCl (PBS) was injected intraperitoneally. Twenty-four hrs later mice were perfused with heparinized saline followed by AZF fixative for 10 min. Brains were stored in AZF for 2 hrs, then placed in cold 15% sucrose overnight. Brains were sectioned on a cryostat at 6–20 µm, coverslipped and immediately examined by fluorescence microscopy using a rhodamine filter.

## Results

To determine whether choroid plexus tumors express a protein with the properties of folate receptors, tumor membranes were examined by Western blotting with an antiserum that was raised against purified mouse FRs [33]. Flow cytometric analysis was used to confirm the specificity of this antiserum, which binds to FR-positive cell lines (LL3, F2-MTXrA, and Mel La) but not FR-negative cell lines (L1210 and Mel) (data not shown).

A major immunoreactive protein of 38 kDa was identified in the tumor but not in brain cortex that surrounded the tumor (Figure 1). This component corresponds to the 38 kDa FR previously identified on mouse cell lines by affinity labelling with NHS-[$^3$H]-folic acid [33]. A less prominent band of ap-



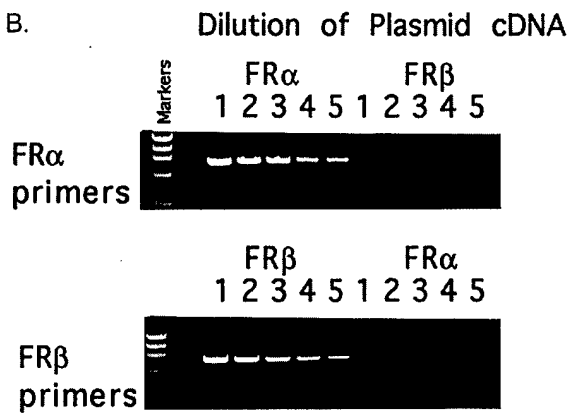




*Figure 4.* Specificity of probes and primers used in the detection of FR-α and FR-β transcripts. *A.* Probes. [α-$^{32}$P]-labeled probes to FR-α and FR-β were tested in Northern blots of mRNA from previously characterized murine cell lines. *B.* Primers. PCR primers for FR-α and FR-β were reacted with plasmid containing cDNAs for FR-α and FR-β, serially diluted by ten-fold (5 ng – 500 fg for lanes 1–5), demonstrating specificity over a five order of magnitude range.

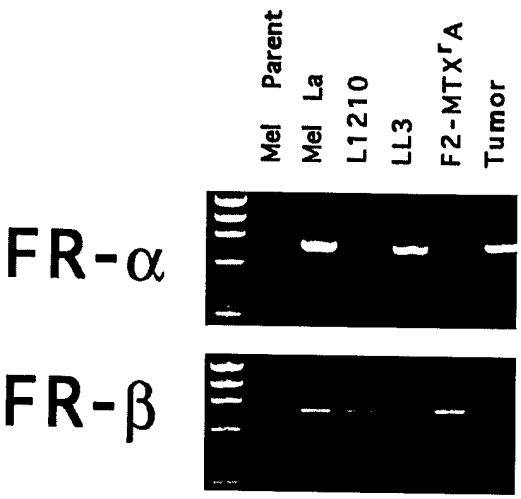



*Figure 5.* Detection of FR-α and FR-β transcripts in mouse tumor cell lines and tumor by RT-PCR. Total RNA from cell lines and tumor (5 μg from each) was reverse transcribed to generate cDNA. An aliquot from each sample cDNA reaction (1 μl) was amplified for FR-α or FR-β by PCR (35 cycles) using primers specific to each respective isoform. FR-α transcript is detectable by RT-PCR in L1210, LL3, Mel La and tumor as a 765 bp band. FR-β transcript is present in L1210, LL3, F2-MTX$^r$A, Mel La, and tumor as a 753 bp band.

proximately 30 kDA was also detected in tumor samples. This component corresponds to the nonglycosylated form of the FR, as previously identified by Brigle et al. [33].

Autoradiographic analysis of [$^{125}$I]folic acid binding to tissue sections of tumor and normal brain parenchyma showed that binding of labeled folic acid was high in the tumor (Figure 2A). Quantitation of silver grain density over tumor tissue and nonlinear regression analysis of the saturation curve indicated that the folate binding by the tumor is of high affinity, with a Kd of 1 nM (Figure 2B).

Regions of normal choroid plexus epithelium were also observed to bind significant amounts of labeled folic acid in both tumor bearing SV11 animals and C57 controls. Normal brain parenchyma had very little binding of labeled folic acid, with less than 1% of the silver grain density of the tumor or normal choroid plexus.

Immunohistochemical staining of normal murine choroid plexus with anti-FR antibody showed a highly polarized distribution of FR (Figure 3A). Reactivity was much more intense on the apical surface of the choroid plexus epithelium. In addition, a dilution of primary anti-FR antibody also demonstrated a marked disparity in the density of FR on the apical surface compared to the basal surface. For example, staining of the basal surface was barely noticeable at a 1 : 5,000 dilution of primary antibody (Figure 3A), yet staining of the apical surface persisted even at a 1 : 50,000 dilution of antibody (data not shown). The normal ependymal lining of the ventricles was also lightly stained, but FR was not detectable in normal brain parenchyma.

Immunohistochemical analysis of tumor tissue indicated a diffuse staining of FRs over the entire cell surface (Figure 3C). Since tumors in the SV11 mice arise from choroid epithelium, the FR staining pattern suggests that the individual neoplastic cells lose their original polarity. Qualitative analysis at various antibody concentrations indicated that FR density is greater on the apical surface of normal choroid epithelium than on the tumor cells (for example, at a 1 : 5,000 dilution, nearly all tumor cells were stained, but at 1 : 50,000 only rare tumor cells were stained even though the apical surface of choroid plexus was stained).

### *Determination of FR probe and primer specificity*

The approaches described above do not distinguish between the α and β isoforms of the FR. To examine whether either α or β or both forms are expressed by tumor, RT-PCR of tumor and normal choroid plexus was performed. The specificities of the primers for PCR and the probes used in the post-PCR Southern blots were evaluated. Northern blot analysis was performed with FR cDNA probes to establish probe specificities. Transcripts for FR-α were detected in the LL3 and Mel La cell lines (Figure 4A). LL3 and Mel La are cell lines known to express the FR-α isoform [13]. Tanscripts from the two cells lines were of expected size (approximately 1700 bp for LL3 and ~ 2000 bp for Mel La). The transcript for FR-β was detected in the cell line F2-MTX$^r$A at an expected size of approximately 1600 bp (Figure 4A).

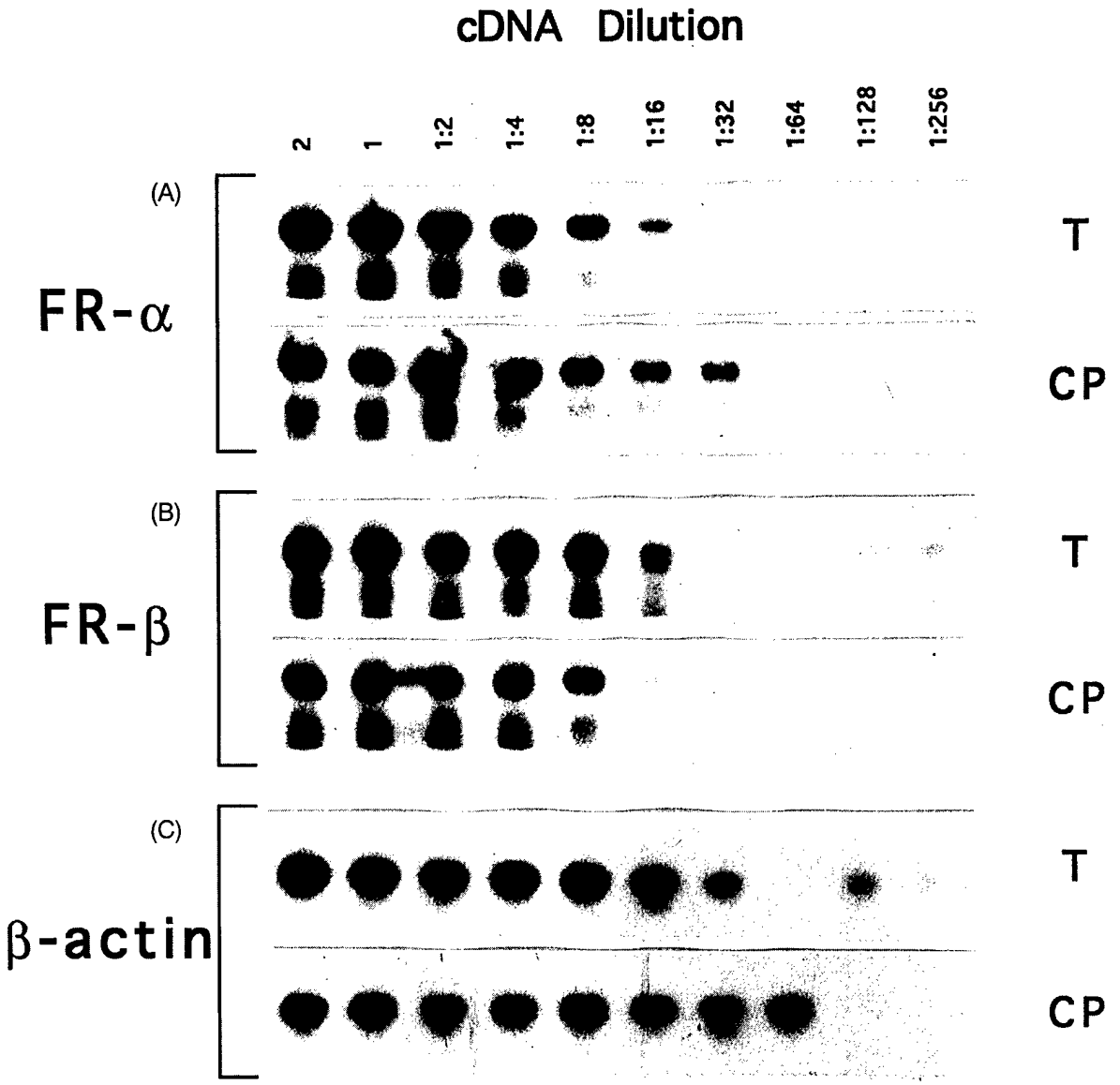



*Figure 6.* Detection of FR-α and FR-β transcripts in mouse choroid plexus tumor and normal choroid plexus by RT-PCR and Southern blot. Total RNA was isolated from choroid plexus (tissue pooled from eight mice) and tumor (0.5 μg RNA from each) by differential centrifugation, and reverse transcribed to generate cDNA. Aliquots of the cDNA reactions (2 μl and dilutions to 1/256 μl) were amplified for FR-α, FR-β, or β-actin by PCR (35 cycles). Amplified products (10 μl) were electrophoresed on a 1.5% agarose gel, transfered to a nylon membrane, and detected with an [α-$^{32}$P]-labeled probe to FR-α (*A*), FR-β (*B*), or β-actin (*C*). Membranes were exposed for 44 hrs. Counts were quantitated on a PhosphorImager following a 12 hr exposure.

For PCR, primer pairs for the coding region of each FR isoform were synthesized and their sensitivity and specificity examined. Various amounts of plasmid containing cDNA for each FR isoform (5 ng – 500 fg) were amplified by PCR in the presence of each set of FR primers. An aliquot of each

reaction product was electrophoresed in an agarose gel and visualized by ethidium bromide staining. Each primer pair was found to be sensitive to at least 500 fg and specific to amplifying its respective isoform at 5 ng (Figure 4B). Additional experiments have shown these primers to be specific up to 1 μg of cDNA-containing plasmid (data not shown). Thus the specificity of the primers extends over at least six orders of magnitude.

*Detection of FR-α and FR-β transcripts in tumor and choroid plexus by RT-PCR*

Due to the very small quantities of total RNA derived from choroid plexus (2 μg) and the relative insensitivity of Northern blots, RT-PCR was utilized to examine FR isoform expression in choroid plexus and tumor. The RT-PCR assay was tested on cDNA derived from five cell lines expressing varying amounts of the FR isoforms. Consistent with the Northern blot analysis, FR-α was present in LL3 and Mel La (Figure 5). In addition, L1210 also contained a detectable amount of FR-α transcript (Figure 5). FR-β was detected in all cell lines examined except the parental Mel line which is known not to express FRs of either isoform (Figure 5). Both FR-α and FR-β were detectable in tumor (Figure 5). RT-PCR followed by Southern blotting of the PCR reaction products detected both α and β FR isoform transcripts in tumor and in normal choroid plexus (Figure 6).

*Analysis of the blood-CSF barrier*

Because the immunoreactive FR staining of the apical surface of normal choroid plexus brought into question the usefulness of the FR as a target on tumor cells, we investigated the status of the blood-CSF barrier in SV11 mice. In non-tumor-bearing mice, macroscopic and microscopic observation of Evans Blue dye indicated that the blood-brain and blood-CSF barriers were intact (data not shown). The median eminence of the hypothalamus, a region of the brain where the BBB is incomplete, showed intense staining.

In tumor-bearing SV11 mice, fluorescence microscopic analysis of the blood-CSF barrier revealed that the stroma of normal choroid plexus stained intensely with Evans Blue, reflecting the rich supply of capillaries. The choroid epithelium itself displayed a moderate degree of staining, but the apical surface was not stained (Figure 3E). CSF and adjacent brain regions lacked staining, indicating that the blood-CSF barrier is still intact in nonaffected regions of the choroid plexus. Microscopic analysis showed intense staining throughout the tumors (Figure 3F).

## Discussion

In this report, endogenously arising brain tumors of SV40 transgenic mice were evaluated for the expression of a recently described tumor-associated antigen, the high affinity folate receptor. Various independent approaches were used to show that these tumors express high affinity folate receptors with properties that are similar to the human receptor. These properties included its molecular weight ($\sim$ 38 kDa) and a binding affinity ($K_D$) for folate of $\sim$1 nM. RNA transcripts of both FR-α and FR-β isoforms were detected in the tumor.

The interest in FR expression by the SV-40-induced tumors stems from several recent findings regarding the use of FR as a target for therapy. First, human choroid plexus tumors and ependymomas, which may have a similar etiology to the transgenic mouse tumors, express FR at substantial levels [11, 12]. Thus, it seemed possible that mouse choroid plexus tumors may also express FR. Second, monoclonal antibodies to the human FR have recently been studied as possible therapeutic agents either as immunotoxins, radiolabelled antibodies, or bispecific antibodies that target tumor cells for lysis by cytotoxic T lymphocytes [28, 29]. Some of these agents have already reached clinical trials with ovarian carcinomas, of which approximately 98% express FR. We have also recently developed a novel targeting agent that consists of folic acid directly conjugated to an anti-T cell receptor antibody [30]. Finally, the endogenously derived-SV40 tumors can be used as a model that more closely mimics the human disease

than do animal studies that involve human tumor transplantation into immune deficient mice.

As with other tumor-associated antigens, the usefulness of FR as a therapeutic target depends in part on its expression in normal tissue. Earlier work with human tissues has indicated that FR-α is expressed to some degree in placenta, epithelia of the choroid plexus, epididymus, lung, thyroid, and kidney proximal tubules, ductal epithelia of the breast and pancreas, and acinar cells of the breast and salivary gland [11, 12, 24]. In the present study, *in situ* analysis revealed high levels of folate binding activity and FR not only in tumor cells but in normal choroid plexus of both control mice and SV11 mice.

One of the findings described here lends support to the notion that FR on tumor cells may be a useful target despite the presence of FR in normal choroid plexus. Immunohistochemistry shows that the FR expressed by choroid plexus is highly polarized. The protein is present on the apical surface of choroid plexus cells on the CSF side of the blood-CSF barrier. This surface may be less accessible than the tumor to blood-borne therapeutic agents. This possibility is supported by evidence that Evans Blue dye was able to stain cells throughout tumor and the stroma of the choroid plexus, but not the apical aspect of the choroid plexus epithelium.

Another possible approach to targeting tumor cells, rather than normal choroid plexus, would involve identifying whether the FR isoforms are differentially expressed. In this regard, the present results are the first characterization of isoforms of FR in any normal murine tissue. FR-α and FR-β were detected in both tumor and normal choroid plexus, but there appeared to be at least a several-fold higher expression of FR-β mRNA in tumor than FR-β mRNA in choroid plexus, based on densitometry of the probe signal and standardization to the β-actin signal. The levels of cell surface FR-α and FR-β protein on tumor cells and choroid plexus remain to be determined when appropriate monoclonal antibodies that distinguish the murine isoforms become available. However, the suggestion that FR-β may be relatively higher in some tumor cells may warrant the search for agents that target this form specifically.

Recently there have been a number of possible therapeutic agents developed that could target cells that bear folic acid receptors without the use of monoclonal anti-FR antibodies. These include folic acid analogs and folic acid conjugates that are directly cytotoxic or that deliver cytotoxic agents [26, 27, 30]. Preliminary results with a folic acid/antibody conjugate injected intravenously into SV11 mice showed that it accumulated preferentially in the tumor as opposed to other regions of the brain (E.J.R., T.A.P., D.M.K., unpublished data). This agent, along with other folic acid conjugates [34], can be used in the SV11 mice to test the relative effectiveness and potential toxicity of various therapeutic regimens.

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# Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells

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# Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells

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Bispecific antibodies that bind to a tumor antigen and the T cell receptor (TCR) redirect cytotoxic T lymphocytes (CTL) to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the folate receptor (FR), is expressed on most ovarian carcinomas and some types of brain cancer. Recently, it was shown that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high-affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein. In this paper, it is shown that the size of these conjugates can be reduced to the smallest bispecific agent yet described (30 kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody KJ16. The scFv/folate conjugates are as effective as IgG/folate conjugates in mediating lysis of $FR^+$ tumor cells by CTL. The optimal folate density was in the range of 5−15 folate molecules per scFv or IgG molecule, which yielded half-maximal lysis values ($EC_{50}$) of approximately 40 pM (1.2 ng/mL for scFv). Finally, the scFv/folate conjugates could efficiently target tumor cells even in the presence of free folic acid at concentrations that are normally found in serum. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors and in reduced immunogenicity.

## INTRODUCTION

It has been known for over 50 years that the immune system is capable of attacking and eliminating very large tumor burdens but sometimes fails to do so (*1*). Although the basis of this "escape" is incompletely understood (*2*), one mechanism involves the failure of tumor cells to express antigens in a context that is essential for recognition by the immune system [reviewed by Pardoll (*3*)]. Another mechanism might be the loss of costimulatory ligands and adhesion molecules that aid in the recognition and activation of T cells (*4*).

One potential way to direct T cells or other immune effector cells against tumor cells is with bispecific antibodies [reviewed by Fanger (*5*)]. Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of a cytotoxic T cell (e.g. TCR[1]). Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). By bringing together the tumor cell and an activated T cell, bispecific antibodies can redirect the cytotoxicity of T cells against tumors. Previous work has demonstrated the effectiveness of bispecific antibodies against tumors *in vitro* and *in vivo* and some clinical trials have been initiated (e.g. see refs 6−12). It has generally been agreed that optimizing the properties of bispecific antibodies should improve their clinical effectiveness.

Among the tumor antigens targeted with bispecific antibodies has been the high-affinity folate receptor (FR), also called the folate binding protein. The FR is now known to be expressed at elevated levels on many human tumors, including ovarian carcinomas [e.g. one study showed that 98% of ovarian tumors express the FR (*13*)], choroid plexus carcinomas, and ependymomas (*14*, *15*). These cancers affect a significant segment of the population: ovarian cancer is the fourth leading cause of cancer death among women (*16*) and at least 30% of early childhood tumors are diagnosed as ependymomal or choroid plexus tumors (*17*, *18*).

The presence of high levels of FR on human tumor cells has made it an attractive candidate for tumor-specific therapeutics. Monoclonal antibodies to the human FR have been generated and shown to be effective at targeting $FR^+$ tumors *in vitro* (*19*−*21*). Clinical trials with radiolabeled antibodies and anti-FR/anti-CD3 bispecific antibodies have recently been initiated (*9*, *10*, *12*). Another approach has been to use the endocytic properties of the FR to deliver toxins or antisense nucleotides to the interior of malignant cells (*22*, *23*). Although relatively low levels of FR mRNA have been detected in most normal human tissues (*14*), several studies have shown that normal choroid plexus, kidney, thyroid, colon, and placenta may have elevated levels [*14*, *24*, *25*; reviewed by Antony (*26*)]. Despite the presence of FR on normal human tissue, clinical trials using the anti-FR/anti-CD3 bispecific antibody have not demonstrated any toxicity associated with cytolysis of normal FR expressing tissue (*9*, *10*).

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[1] Abbreviations: CTL, cytotoxic T lymphocyte; FR, folate receptor; TCR, T cell receptor; scFv, single-chain antibody binding domain; MHC, major histocompatibility complex; $K_D$, dissociation constant; SV40, simian virus 40; $EC_{50}$, concentration of antibody/folate conjugate required for half-maximal CTL mediated cytotoxicity; fol, folate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; FITC, 5-aminofluorescein isothiocyanate; Ga, gallium; Fab, antigen binding fragment derived from papain digestion of Ig molecule; V regions, variable regions of IgG heavy and light chains; $\epsilon$, extinction coefficient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.3; PBS-BSA, PBS containing 0.1% bovine serum albumin; MTX, methotrexate.

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The high intrinsic affinity of folate for FR ($K_d \sim 1$ nM) suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR$^+$ tumor cells for lysis by T cells. We recently reported that such conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein [$K_d \sim 1.5 \times 10^{-6}$ (*27*)], which is responsible for normal dietary uptake of folate and the transport of folate-based dihydrofolate reductase inhibitors such as methotrexate. It is reasonable to predict that the most effective agents for targeting solid tumors will have reduced sizes that allow greater tumor penetration. For instance, comparative biodistribution studies with $^{125}$I-labeled IgG, F(ab)$_2$, Fab fragments, and scFv in human colon carcinoma xenografts in athymic mice demonstrated that scFv molecules penetrated tumor more rapidly, to a greater depth, and more uniformly than other forms of the antibody (*28*).

In this paper, a 29-kDa scFv of the anti-V$\beta$8 antibody KJ16 (*29*) was conjugated with folate and its targeting potential was evaluated *in vitro*. Cytotoxicity assays with these preparations showed that lysis of mouse FR$^+$ tumor cells was highly specific and correlated directly with FR density ($r = 0.93$). Comparison between folate-labeled-IgG and scFv demonstrated that both conjugates have nearly identical targeting efficiencies ($EC_{50} = 40$ pM), and lysis with scFv/folate could be detected at concentrations as low as 1 pM. Direct competition experiments with free folate demonstrated that the scFv/folate conjugate could effectively target FR$^+$ tumor cells even at folate concentrations above normal serum levels. The reduced size of the scFv/folate compared to other bispecific reagents as well as its high potency suggests that it has potential for *in vivo* therapy. In addition, the conjugate may serve as a model for the development of future novel bispecific agents that contain small ligands specific for tumor cell surface antigens.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Antibodies.** The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal calf serum, 1.3 mM L-glutamine, 100 units of penicillin/mL, 100 $\mu$g/mL streptomycin, and 50 $\mu$M 2-mercaptoethanol: Mel, murine erythroleukemia cell (*30*); La, a subline of Mel selected on low folate (*31*); L1210, a murine leukemia cell line (*32*); and F2-MTX$^r$A, a MTX-resistant subline of L1210 selected for increased expression of FR-$\beta$ by growth on low folic acid (*33*). La expresses primarily the $\alpha$ isoform of folate receptor (FR), F2-MTX$^r$A expresses only the FR-$\beta$ isoform, and L1210 expresses both $\alpha$ and $\beta$ isoforms. CTL clone 2C, a V$\beta$8$^+$ alloreactive cell line specific for L$^d$ (*34*), was maintained in the same RPMI medium supplemented with 10% (v/v) supernatant from concanavalin A-stimulated rat spleen cells, 5% methyl $\alpha$-mannoside, and mitomycin C treated BALB/c mouse spleen cells as stimulators. KJ16 is a rat IgG antibody specific for the V$\beta$8.1−2 domains of the TCR (*35*) and was provided by Drs. Kappler and Marrack. KJ16 monoclonal antibody was prepared from tissue culture supernatant generated in a Vita-Fiber miniflow path bioreactor (Amicon) and concentrated by precipitating twice in 50% ammonium sulfate. KJ16 Fab fragments, FITC-labeled Fab fragments, and KJ16 scFv were generated and purified as described previously (*29*). Briefly, scFv was refolded from inclusion bodies, and monomeric scFv was purified by G-200 HPLC purification. Monoclonal antibody 30.5.7 is specific for the major histocompatibility complex (MHC) class I product L$^d$ (*36*) and was prepared as ascites fluid and used without further purification in some cytotoxicity assays.

**Preparation of Antibody/Folate Conjugates.** Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (*27*, *37*). Unless indicated, a 5-fold molar excess of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce Chemical Co.) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 min at room temperature in the dark, a 20−700-fold molar excess of the EDC-activated folate was added to 0.1−0.5 mg of antibody in 0.1 M MOPS, pH 7.5. After 1 h at room temperature, the sample was either applied to a Sephadex G-25 column pre-equilibrated in 0.1 M MOPS or immediately dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3). If passed over a G-25 column, the excluded-peak fractions were pooled and dialyzed against PBS. After dialysis, protein concentrations were determined using the bicinchoninic assay (Pierce) using a protein A purified intact mouse antibody as the standard. Antibody conjugates were also analyzed spectrophotometrically at 363 nm, and the density of the folate per antibody was calculated by dividing the molar concentration of folate on the conjugate ($A363/\epsilon_M$; $\epsilon_M = 6197$ M$^{-1}$) by the antibody concentration. Folate densities obtained ranged from ~1 to ~120 folates/antibody. Conjugates were stored at 4 °C in the dark.

**Mass Spectrometry.** Mass spectra were obtained on a TofSpec using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and concentrated to 10−25 pmol/mL. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois.

**Folate Binding Assays.** Binding assays were conducted by using $^{125}$I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.3 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 50 $\mu$L of PBS-BSA for 1 h at 37 °C. Incubation at 37 °C has previously been shown to produce levels of binding similar to that obtained with acid pretreatment (*27*). Samples were loaded into tubes containing 300 $\mu$L of oil [80% (v/v) dibutyl phthalate/20% (v/v) olive oil], and bound and free ligand were separated by a 3 s centrifugation at 12000*g*. Tubes were frozen and cut to allow the radioactivity in the cell pellet and supernatants to be quantitated separately.

**T Cell Receptor Binding Assays.** The relative affinity of the scFv/folate conjugates for the TCR was determined by a competition assay with 5-aminofluorescein isothiocyanate (FITC)-labeled KJ16 Fab fragments as previously described (*29*). In brief, various concentrations of antibody were added to triplicate sets of $6 \times 10^5$ 2C cells in the presence of a constant amount of FITC-labeled Fab fragments. After a 30 min incubation on ice, the entire mixture (antibody + FITC-labeled Fab fragments + 2C cells) was passed through a flow cytometer without washing. Inhibition by various KJ16 preparations was measured by quantitating the decrease in bound fluorescence by flow cytometry (performed with a Coulter Electronics EPICS 752 at the University of Illinois Biotechnology Center). The concentrations of unlabeled antibody giving 50% inhibition ($IC_{50}$) were determined relative to the maximum fluorescence (in the absence of inhibitor) and the background fluorescence (in the presence of a large excess of intact antibody).

**Cytotoxicity Assays.** Tumor cells were labeled with

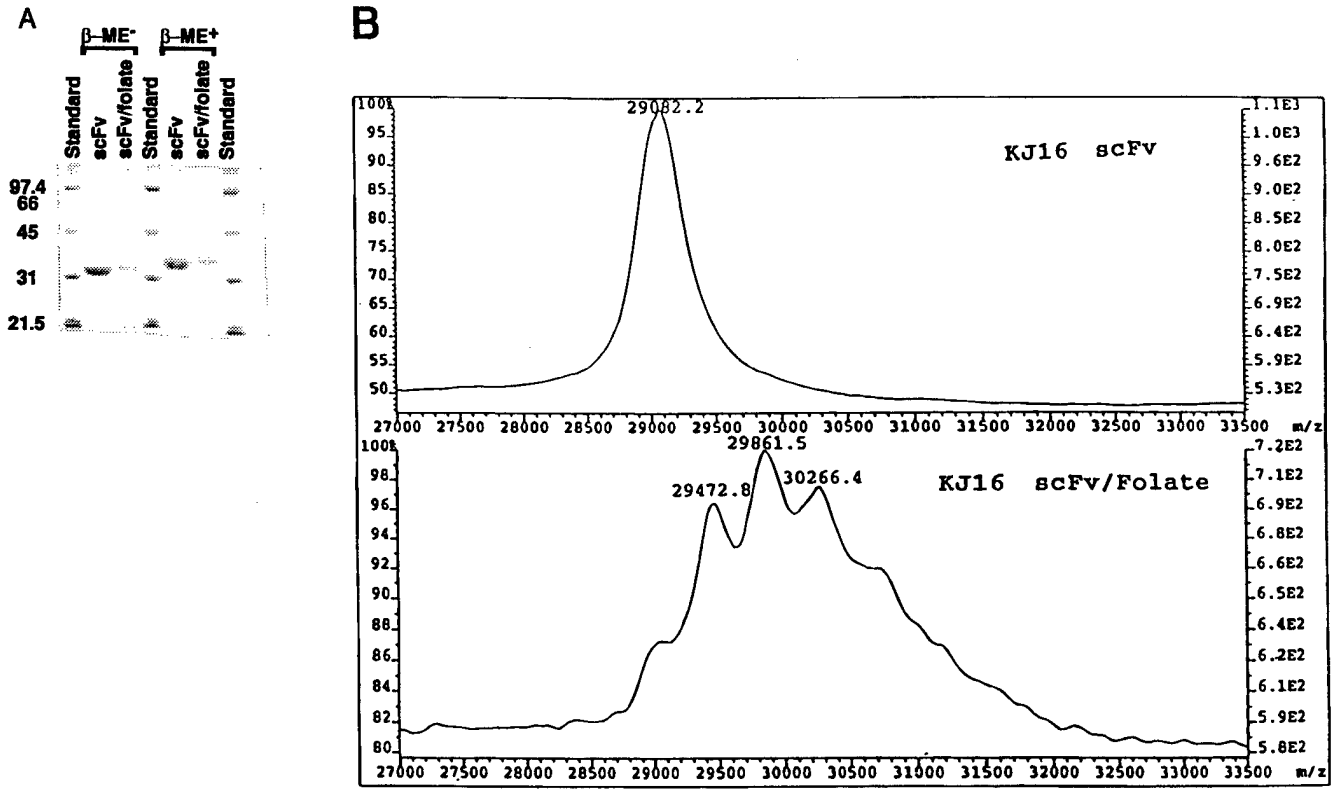


**Figure 1.** SDS–PAGE analysis and mass spectra of purified preparations of scFv KJ16 and scFv/folate conjugate: (A) samples were electrophoresed through a 10% polyacrylamide gel under reducing and nonreducing conditions, and proteins were visualized by staining with Coomassie Blue; (B) samples were concentrated, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and mass spectra were obtained on a TofSpec using electrospray ionization. Purified scFv existed as a single species with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody.

50–100 $\mu$L of $^{51}$Cr (2.5 mCi/mL) for 60 min at 37 °C, washed twice with folate-free RPMI 1640 medium containing 5% (v/v) fetal calf serum (folate-free media), and used in 96-well plate cytotoxicity assays at $10^4$ cells per well. Because each of these cell lines also expressed the alloantigen $L^d$, which is recognized by CTL 2C, assays were performed in the presence of anti-$L^d$ antibody to minimize non-FR-mediated lysis. Ascites of anti-$L^d$ antibody 30.5.7 was diluted 1:100 into folate-free media containing effector cells (2C). Effector cells were added to target cells at an effector-to-target cell ratio of 5:1. Antibodies and folate/antibody conjugates were diluted in folate-free media and added to triplicate wells at various concentrations. Plates were incubated at 37 °C for 4 h in 5% $CO_2$, and supernatants were removed for $\gamma$ counting. For the inhibition of scFv/folate by free folate, a nonsaturating concentration of scFv/folate (3 nM) that would generate maximal cytotoxicity was used together with various concentrations of free folate. Unless indicated otherwise, the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates [e.g. % specific release = (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100].

$EC_{50}$ values (i.e. the concentration of antibody/folate conjugate required for half-maximal specific lysis) were determined by linear regression. Among independent experiments, $EC_{50}$ values and maximum cytotoxicity could vary for the same conjugate preparation primarily due to differences in CTL activity. For example, noticeable reductions in CTL 2C activity can be observed after multiple passages *in vitro* (unpublished data, D.M.K.). Therefore, for comparison of $EC_{50}$ values from different experiments (i.e. Figure 5B), assay results were normalized by dividing each calculated $EC_{50}$ by the $EC_{50}$ of the most potent conjugate in a given experiment [e.g. $EC_{50}^{normalized} = (EC_{50}/EC_{50}^{low})$]. The inverse of this normalized value, $[(EC_{50}^{normalized})^{-1}$, which we have called the targeting index] was plotted as a function of folate density on the antibody, where a targeting index = 1 specifies the most potent conjugate.

## RESULTS

**Characterization of scFv/Folate Conjugates.** The scFv of KJ16, an anti-V$\beta$8 antibody, was purified from *Escherichia coli* inclusion bodies after guanidine denaturation, refolding, and HPLC gel filtration. Purified scFv migrated as an apparent 35-kDa protein on SDS–PAGE gels (Figure 1A). Folate was coupled to the scFv using the carbodiimide (EDC) reaction, which links carboxyl groups of folate to primary amine groups on the protein. In the engineering of the scFv, the $V_L$ and $V_H$ domains were joined by the 26 residue linker, 205s, that contains 8 lysine residues (*29, 38*). We reasoned that the presence of multiple lysine residues in a highly accessible, solvent-exposed region may result in higher folate densities in the linker region as opposed to the antibody V regions. In initial studies, folate was coupled to the scFv at a 100:1 folate to antibody molar ratio. Under these conditions, scFv/folate preparations contained an average of 3–8 folates per antibody ($N$ = 3), on the basis of spectrophotometric analysis. Consistent with this finding, migration of the scFv/folate conjugate on SDS–PAGE gels was slightly slower than that of scFv and the band was more diffuse (Figure 1A).

The folate density determined by photometry does not provide information about the heterogeneity of the conjugates. To determine the range of epitope densities within a single preparation, scFv/folate conjugates were

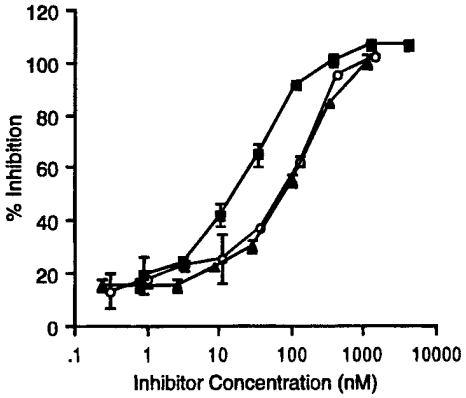


**Figure 2.** Binding of KJ16 scFv/folate to cell surface T cell receptor. The binding of FITC-labeled KJ16 Fab fragments to the V$\beta$8-positive T cell clone 2C was inhibited by purified scFv/folate, scFv, or Fab fragments. A total of $6 \times 10^5$ 2C cells was incubated for 30 min at 4 °C with FITC-labeled KJ16 Fab fragments ($\sim 7.0 \times 10^{-8}$ M) and various concentrations of folate-labeled scFv KJ16 (7.8 fol/scFv, ▲), unlabeled scFv (■), and unlabeled Fab fragments (○). A relative affinity of the scFv/folate was determined by comparing the concentrations required to inhibit 50% of the FITC-labeled Fab fragments from binding the 2C TCR.

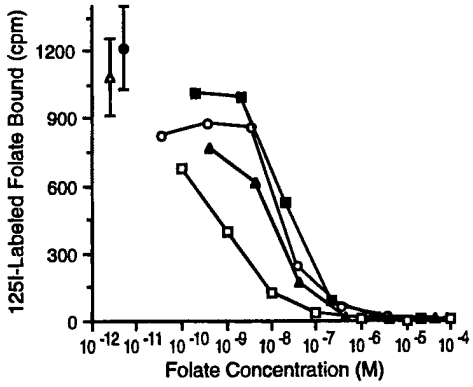


**Figure 3.** Binding of KJ16 scFv/folate to folate receptors. $^{125}$I-labeled folate ($\sim 1.8 \times 10^{-10}$ M, 2000 Ci/mM) was incubated with F2-MTX$^r$A cells in the presence or absence of competitors for 1 h at 37 °C. Concentrations refer to folate rather than antibody concentrations. Competitors included free folate (□) and KJ16 scFv/folate conjugates with different folate densities: 2.8 fol/scFv (○), 9.2 fol/scFv (■), and 20.4 fol/scFv (▲). Inhibition was not observed in the absence of competitor (△) or in the presence of unconjugated scFv (●) (error shown is ± average SEM).

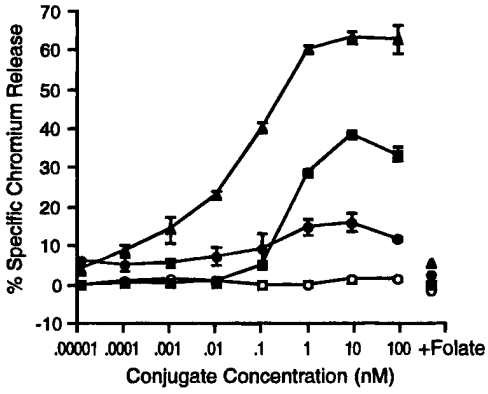


**Figure 4.** Cytotoxicity assay of various tumor cells lines with the scFv/folate conjugate and CTL clone 2C. Various concentrations of the scFv/folate conjugate were incubated with $^{51}$Cr-labeled tumor cells and CTL 2C for 4 h at an effector-to target ratio of 5:1. Experiments were performed in the presence of anti-L$^d$ antibody to minimize lysis due to recognition of L$^d$, the nominal ligand for CTL 2C. Lysis correlated directly with the level of FR expressed by the cell line: F2-MTX$^r$A (200 000 sites/cell, ▲); La (60 000 sites/cell, ■); L1210 (8 000 sites/cell, ●); Mel (not detectable FR, ○). Assays with free folate at a final concentration of 1.5 $\mu$M were performed with the scFv/folate conjugate at a concentration of 0.09 nM (+ folate).

examined by electrospray ionization mass spectrometry (Figure 1B). Unlabeled scFv demonstrated a single peak with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody. Integration of mass spectra showed that >85% of the scFv molecules were labeled with one or more folate molecules. Folate densities estimated from mass spectra were generally 1.5–2-fold lower than densities estimated by spectrophotometry. This could in part be due to lower solubility of high-density conjugates under conditions required for mass spectrometry or to the dissociation of some folate molecules during ionization.

**Binding of scFv/Folate Conjugates to the T Cell Receptor.** To examine if folate conjugation affected the binding of scFv antibodies to the TCR, a scFv/folate conjugate was compared with unlabeled scFv. In a competitive flow cytometric assay, fluorescein-labeled KJ16 Fab fragments were inhibited from binding the V$\beta$8-positive CTL clone 2C by unlabeled Fab fragments, scFv, or scFv/folate (Figure 2). As shown previously, scFv antibodies have an approximate 3-fold higher apparent affinity than Fab fragments, possibly because of the presence of noncovalently associated scFv dimers (*29*). Comparison of folate-labeled and unlabeled scFv showed that the folate conjugate had an apparent affinity ~3-fold lower than scFv (i.e. approximately equal to KJ16 Fab fragments). This decreased binding, compared to unlabeled scFv, could be due either to chemical modification of active site residues with folate or to the interference of dimer formation by folate. The fact that folate conjugates bound the TCR as well as KJ16 Fab fragments, which have a $K_D$ of ~130 nM (*29*, *39*), indicated that the conjugates have potential to mediate lysis of target cells by CTLs.

**Binding of scFv/Folate Conjugates to Folate Receptors on Tumor Cells.** The ability of scFv/folate conjugates to bind folate receptors (FR) on the surface of tumor cells was examined by a competition binding assay using $^{125}$I-labeled folate as the labeled ligand (Figure 3). The competition assay used the F2-MTX$^r$A cell line that expresses the $\beta$ isoform of the FR. Competitors included various concentrations of free folate, unlabeled scFv, and three different scFv/folate preparations. Examination of the binding curves showed that folate-conjugated antibody, but not unlabeled scFv, binds to the FR$^+$ tumor cell line. However, on the basis of molar folate concentration, the folate conjugates had a relative affinity that was approximately 10–30-fold less than that of free folate. This decrease in apparent affinity was consistent with previous observations (*27*) and could be partly attributed to the carbodiimide labeling procedure. This procedure links folate through either the $\alpha$ or $\gamma$ carboxyl group, but only linkage through the $\gamma$ carboxyl retains binding (*40*). It is also possible that receptor-mediated internalization or decreased accessibility of the FR may also explain the lower apparent affinity of the folate conjugate. Included in the latter is the possibility that neighboring folates or amino acids sterically hinder interaction with the FR.

**CTL-Mediated Lysis of FR$^+$ Tumor Cells by scFv/Folate Conjugates.** The specificity and efficiency of tumor targeting with scFv/folate conjugates were examined in a $^{51}$Cr release assay with CTL clone 2C (Figure 4). Four different tumor cell lines that have a range of cell surface FR densities were used as target cells: F2-

MTX$^r$A (200 000 sites/cell); La (60 000 sites/cell); L1210 (8 000 sites/cell); Mel (no detectable FR). Each of the FR$^+$ cell lines was lysed in the presence of the scFv/folate conjugate, and the extent of lysis was directly correlated with the expression level of the FR ($r = 0.93$). Lysis was completely inhibited by free folate, indicating that targeting of the tumor cells was specifically mediated by the folate receptor and not some other cell surface molecule. The lysis mediated by these conjugates was highly specific (e.g. the FR$^-$ cell line Mel was not lysed by the conjugate even at concentrations over $10^5$ times that required for detectable killing of the FR$^+$ cell line F2-MTX$^r$A) and extremely potent (e.g. lysis was detectable at concentrations as low as 1 pM of scFv/folate). The slight reduction in observed lysis at the highest concentrations of conjugate occurs because excess bispecific antibody yields monospecific binding without cell-to-cell cross-linking (*41*). The presence of reduced folate carrier protein (as present in Mel and in all other normal cells) does not result in cell destruction. It is important to point out that the FR density reportedly on ovarian tumors is even higher (~1 million/cell) than those on these tumor cell lines (*21*).

**Effects of Folate Density on Targeting.** To examine the effects of folate density and labeling on the targeting efficiency of scFv/folate conjugates, the antibody was labeled with folate under various carbodiimide-mediated coupling conditions. The carbodiimide EDC couples folate through the free carboxyl groups, but when used in the presence of protein, it may also lead to protein modification and subsequent precipitation or inactivation. To evaluate the optimal levels of EDC for folate coupling, several different concentrations of EDC at a constant folate concentration were used during coupling. EDC used at either 13 or 65 mM generated conjugates with approximately equal targeting efficiency. EDC used at 260 mM yielded conjugates with reduced efficiency and frequently led to protein precipitation (data not shown).

To directly examine the effect of folate density, activated folate was prepared at a constant EDC/folate ratio (5:1) and conjugates were produced by adding different ratios of activated folate to the scFv protein. After dialysis to remove unreacted folate and excess EDC, conjugates were evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency. Folate densities ranged from approximately 1 to 20 folates per scFv. As shown in Figure 5A, each of the conjugates was capable of mediating lysis of the FR$^+$ tumor cell line by CTL clone 2C. However, conjugates with either the lowest density (1.3 folates/scFv) or highest density (20.4 folates/scFv) were 5−10-fold less effective than conjugates with intermediate folate densities.

To better evaluate the effects of folate density, the above experiment was performed with several additional folate conjugate preparations. To directly compare the results of these assays, the $EC_{50}$ of each conjugate was determined by linear regression and divided by the $EC_{50}$ of the conjugate that yielded the highest targeting efficiency (i.e. the lowest $EC_{50}$; see Experimental Procedures). A plot of the normalized targeting values as a function of the folate density (Figure 5B) demonstrates that optimal folate density appears to be in the range of 5−15 folates/scFv. At higher folate densities, the targeting efficiency of the conjugates is generally lower and more variable than those of conjugates with an intermediate amount of folate. The reduction in targeting efficiency at higher folate densities is not due to the inability of these conjugates to bind to FR$^+$ cells (Figure 3), but is likely a consequence of chemical modification

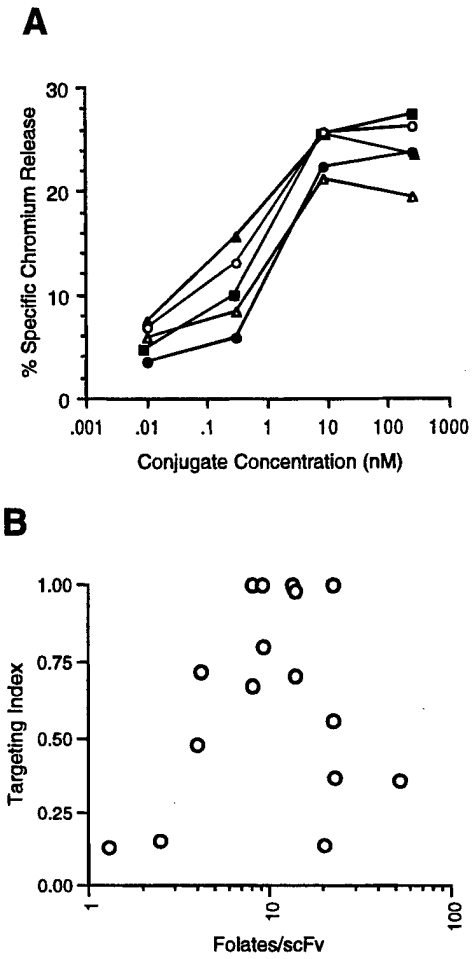


**Figure 5.** Effect of folate density on targeting with scFv/folate conjugates: (A) scFv/folate conjugates with different folate densities were prepared by labeling scFv protein with various amounts activated folate, using an EDC/folate ratio of 5:1 (EDC = 65 mM). Folate densities were 1.3 fol/scFv, ●; 4.0 fol/scFv, ■; 9.2 fol/scFv, ▲; 9.3 fol/scFv, ○; and 20.4 fol/scFv, △. Conjugates were tested in cytotoxicity assays using F2-MTX$^r$A and CTL 2C. (B) Relative targeting efficiency of various scFv/folate conjugates as a function of increasing folate density. Targeting index values were determined by normalizing $EC_{50}$ values of the various conjugates with the most effective conjugate (value = 1) (see Experimental Procedures for details). Each open circle (○) represents a single scFv/folate conjugate prepared using an EDC/folate ratio of 5:1 (EDC 33−65 mM) and tested in cytotoxicity assays using F2-MTX$^r$A and CTL 2C.

of amino acid residues important in TCR binding by the scFv or in scFv stability.

**Comparison of Intact Antibody and scFv.** To determine the relative effectiveness of intact KJ16 IgG versus scFv-KJ16, both forms were labeled with folate at a 100:1 molar ratio of activated folate to antibody and under identical EDC reaction conditions (33 mM EDC, 6.7 mM folate). These conditions yielded folate densities of 7 and 5 for the scFv and intact antibody, respectively. Cytotoxicity assays with these preparations showed nearly identical targeting efficiencies for the intact and scFv forms of KJ16 (Figure 6). The concentration required to obtain 50% of the maximal specific release ($EC_{50}$) was approximately 40 pM (1.2 ng/mL for scFv). Comparison of intact and scFv conjugates in an $^{125}$I-labeled folate binding assay indicated no significant difference in their ability to bind FR (data not shown).

Intact KJ16 antibody was also labeled at various folate densities to determine if targeting efficiency could be optimized further. Conjugates were again evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency (Fig-

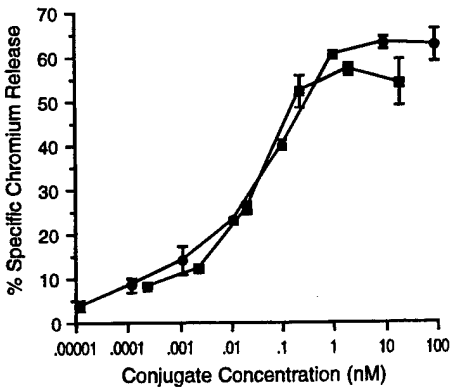


**Figure 6.** Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 mM EDC, 100:1 molar ratio of folate/antibody), yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using $^{51}$Cr-labeled F2-MTX$^r$A cells and CTL clone 2C.

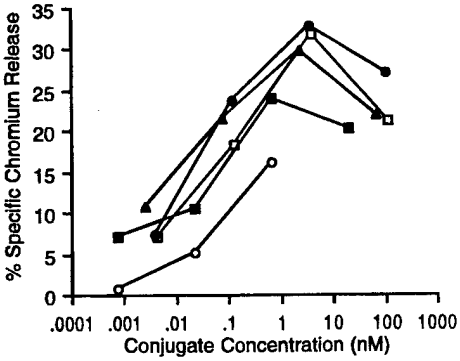


**Figure 7.** Effect of folate density on targeting with IgG/folate conjugates. Intact antibody was labeled at several folate concentrations to determine if, like scFv, a specific range of folate density would yield the optimal targeting effectiveness. Conjugates with the following densities were assayed with $^{51}$Cr-labeled F2-MTX$^r$A cells and CTL clone 2C: 4.5 fol/IgG, □; 7.0 fol/IgG, ●; 13.6 fol/IgG, ▲; 56.7 fol/IgG, ■; and 126.2 fol/IgG, ○.

ure 7). Folate densities ranged from approximately 4−126 folates/antibody molecule. As shown with scFv preparations, high and low folate density resulted in decreased targeting effectiveness for the intact antibody. As with scFv preparations, the optimal densities appear to be in the range of 5−15 folates/antibody, as determined by spectrophotometry.

**Inhibition of scFv/Folate Conjugate-Mediated Lysis by Free Folate.** Normal serum folate may reduce the effectiveness of the scFv/folate conjugate by competing for the folate receptor *in vivo*. A $^{51}$Cr release assay was used to evaluate the effectiveness of the scFv/folate conjugate-mediated lysis at biologically relevant concentrations of free folate (Figure 8). Free folate was diluted in folate-free media, and various concentrations were added to triplicate wells containing $^{51}$Cr-labeled F2-MTX$^r$A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (3 nM) that would generate maximal specific release. The resulting titration curve demonstrated that at normal human serum folate levels [9−36 nM (*42*, *43*)] the scFv/folate conjugate retained most of its activity. For instance, at 20 nM folate, the scFv/folate conjugate exhibited over 80% of its CTL-mediated targeting potential. Although murine serum folate levels are significantly higher than normal human serum values (124−700 nM) due to folate-rich chow (*44*, *45*), even at these elevated folate concentrations, the scFv/folate conjugate exhibited 30−60% of its targeting potential.

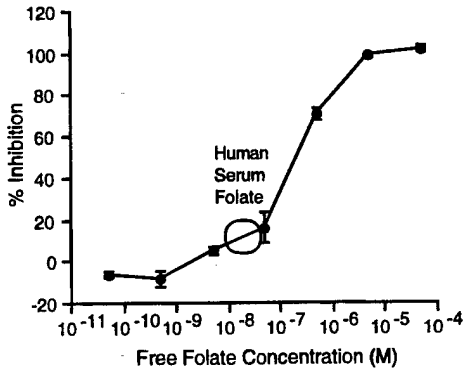


**Figure 8.** Inhibition of scFv/folate-mediated lysis by free folate. Various concentrations of free folate were added to triplicate wells containing $^{51}$Cr-labeled F2-MTX$^r$A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (~8 fol/scFv) that would yield maximal specific release (3 nM). Inhibition was calculated as a function of the specific release in the absence of folate competitor. Normal human serum folate concentrations range from 9 to 36 nM (*42*, *43*).

## DISCUSSION

The most effective agents for targeting solid tumors will likely have reduced sizes that allow greater tumor penetration. This paper characterizes the smallest bispecific agent yet described for redirecting the activity of immune effector cells against tumors. Initial bispecific antibody studies to target ovarian tumors that express high-affinity FR have used intact heterobifunctional antibodies (~150 kDa) that bind to CD3 and the FR. These agents showed efficacy in animal models, and they have recently entered testing in clinical trials (*9*, *10*, *12*). Although not yet reported for anti-FR antibodies, several laboratories have shown that it is possible to engineer smaller bispecific antibodies of ~60 kDa by linking two scFv regions (*41*, *46*, *47*). Here we show that the size of a bispecific targeting agent can be reduced even further to ~30 kDa for the scFv/folate conjugates. Furthermore, the targeting efficiency of the engineered scFv/folate conjugate is comparable to that of the native intact antibody/folate conjugates.

For coupling of folate to the anti-V$\beta$8 scFv KJ16, a carbodiimide reaction that links the carboxyl groups of folate to the amino groups of the antibody was used. KJ16 scFv binds to the cell surface TCR with an affinity that is similar to that of the intact KJ16 antibody (*29*). The $V_L$ and $V_H$ regions of the scFv are linked by a 26 amino acid region that contains 8 lysine residues. We reasoned that this charged linker would be accessible to folate, would be located distal to the binding site, and would contain multiple attachment sites for folate through the EDC reaction. Under coupling conditions where folate concentrations are nonsaturating, the scFv and intact antibody have comparable densities of folate attached per molecule (~5−15 folates/molecule), despite the 5-fold greater size of the intact antibody. Cytolytic assays demonstrated that these conjugates have very similar targeting efficiencies (Figure 6).

Although folate is probably attached to lysines present in the linker, the scFv/folate conjugates are actually heterogeneous populations as evidenced by mass spectra (Figure 1B). Within a scFv/folate preparation there are most likely conjugates with enhanced targeting properties and conjugates with diminished targeting properties. The former could include not only those with folate at an accessible location but perhaps those with multiple

folates that allow for multivalent interactions with FRs on tumor cells. Conjugates which have diminished bispecific properties would include those that have folate attached through amine groups in the scFv active site or in regions which destabalize the $V_L-V_H$ interaction. These are analogous to those preparations derived with very high folate densities, where the targeting efficiency is significantly reduced (Figures 5 and 7).

Several considerations suggest that further optimization of the scFv/folate conjugates could yield even more potent agents. The anti-TCR antibody KJ16 has a relatively modest $K_D$ of ~100 nM (*29*). Our laboratory is currently engineering higher affinity anti-TCR antibodies by antibody display methods; antibodies with $K_D \leq 1$ nM have been routinely generated using similar approaches (*48*). In addition, the affinity of the scFv/folate conjugate for the $FR^+$ tumor cells was up to 30-fold less than the affinity of free folate for the FR (Figure 3). Coupling of folate through the $\gamma$ carboxyl, plus homogeneous linkage perhaps through cysteine or multiple cysteines, should improve the affinity for the FR. Alternatively, other folate analogs with higher affinity than folate could be employed (*49*). The use of these strategies should allow the development of scFv conjugates that have $EC_{50}$ values considerably less than those described in this paper.

It is of significant note that the scFv agent described here not only is the smallest agent but is at least as potent *in vitro* as bispecific antibodies described in the literature. For example, the $EC_{50}$ of other bispecific agents range from 1 to 100 ng/mL (*21*, *50*–*52*). Various other antibody or folate-based targeting agents have $EC_{50}$ values that range from 0.1 to 200 ng/mL (e.g. refs 37, 40, 53–56). *In vitro* assays for these other agents typically involved 24 h incubation periods, while the cytotoxicity assays for CTL-mediated lysis, described here and elsewhere, are 4 h incubations. Thus, there is reason to believe that the scFv/folate conjugate has considerable promise *in vivo*: It has an $EC_{50}$ of approximately 1 ng/mL, is smaller than the other agents, and remains effective at folate levels found in normal human serum. The scFv/folate targeting effect was inhibitable by free folate, but only at concentrations that were considerably higher (>1000-fold) than the folate conjugate concentration (Figure 8). Schodin *et al.* (*57*) have previously shown that <1% of the total number of TCRs per CTL need to be triggered for CTL-mediated cytolysis to occur. Thus, it is possible that >90% of the folate/antibody conjugates were inhibited from binding the target cell but sufficient conjugate remained bound to trigger maximal lysis.

The fact that the scFv/folate conjugate is in direct competition with serum folate also brings about the intriguing possibility of modulating the effectiveness of scFv/folate conjugate treatment by altering the levels of serum folate. For instance, recent studies with mice have shown that serum folate can be intentionally decreased up to 100-fold with special low-folate diets (*44*, *45*). The decreased folate concentration greatly enhanced the ability of a $^{67}$Ga-labeled deferoxamine/folate conjugate to image $FR^+$ tumors *in vivo* (*44*). We envision that similar low-folate diets will likewise enhance the therapeutic effectiveness of the scFv/folate conjugate. Conversely, serum folate could be increased in situations where nonspecific T cell interactions lead to adverse side effects. Thus, the use of folate as the small molecule ligand specific for a tumor antigen may allow for additional levels of regulation normally not available with other immunotargeting agents.

*In vivo* tests to compare scFv and intact folate conjugates are currently underway in SV40 transgenic mice that develop choroid plexus tumors exhibiting elevated levels of the high affinity folate receptor (*58*). Preliminary results indicate that T cells specifically infiltrate the tumor after treatment with the scFv/folate conjugate (B.K.C., T.A.P., D.M.K., and E.J.R., unpublished data). At this time, it is unclear whether conjugate-bound T cells extravasate into the tumor or if T cells first extravasate into the tumor and subsequently recognize the conjugate bound to cell surface FR. The latter mechanism would favor the enhanced tumor penetration characteristics of the smaller scFv molecule and would likely lead to increased therapeutic effectiveness.

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# Antigen Recognition and Allogeneic Tumor Rejection in CD8$^+$ TCR Transgenic/RAG$^{-/-}$ Mice[1]

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**Three sources of help for the development of a CD8$^+$ CTL response have been described: the CD4$^+$ direct and indirect pathways and the CD8$^+$ direct pathway. In an effort to understand the minimal requirements for the development of a CTL response in vivo, we have bred mice transgenic for the 2C TCR onto a RAG$^{-/-}$ background. The 2C T cells in this animal are exclusively CD8$^+$ CTLs of a single specificity, and they exhibit altered thymic maturation compared with that of T cells from 2C TCR/RAG$^{+/+}$ mice. T cells from 2C TCR/RAG$^{-/-}$ mice can be activated to a high level in vivo by administration of a self-MHC-restricted antigenic peptide. The 2C TCR/RAG$^{-/-}$ mice are able to reject B7-negative allogeneic tumors bearing the appropriate peptide/MHC ligand p2C/L$^d$. These mice fail to reject syngeneic tumors, and their RAG$^{-/-}$ littermates lacking 2C T cells uniformly succumb to both allogeneic and syngeneic tumors. Moreover, blockade of B7 costimulatory molecules fails to prevent tumor rejection in the 2C TCR/RAG$^{-/-}$ mice, suggesting that allorejection is occurring independently of B7-mediated costimulation as well as in the absence of CD4$^+$ T cells. CTLs isolated from the site of the tumor during the period of rejection express the activation marker CD25 and are able to mediate ex vivo cytolysis of tumor cells bearing the appropriate Ag. These results suggest that in this TCR transgenic model with a very high precursor frequency, CTL development can occur in the absence of B7:CD28 costimulation and without CD4$^+$ help.** *The Journal of Immunology,* **1997, 159: 4665–4675.**

Transplantation of tissue containing mismatched MHC Ags generally produces a vigorous alloreactive response. The strength of such a response has been attributed to a high frequency of T cells (~10%) capable of recognizing alloantigen. While pathogen-specific T cells generally recognize a foreign peptide in the context of self-MHC, alloreactive T cells usually recognize self peptides in the context of a foreign MHC molecule. Three possible sources of help have been described in the generation of an alloreactive CTL response: 1) the direct CD4$^+$ pathway, 2) the indirect CD4$^+$ pathway, and 3) a direct CD8$^+$ pathway (1). Where it has been examined in tumor models, an additional CD8$^+$ indirect pathway is predominant in CTL development (2, 3). The direct CD4$^+$ pathway involves Th cells that recognize antigenic peptide/MHC-II complexes on donor cells. The indirect CD4$^+$ pathway involves Th cells that recognize peptides derived from donor class I MHC presented by self-MHC class II molecules on self-APCs (4). The direct CD8$^+$ pathway involves CTL recognition of peptide/MHC class I complexes on donor cells and is considered to be highly dependent on the number of donor professional APCs in a graft (5). The CD8$^+$ indirect pathway consists of initial CTL cross-priming by host bone marrow-derived APCs and requires the uptake and presentation of tumor Ags on host MHC class I molecules (2, 3). The relative importance of the various sources of help for CTL activation is not fully understood, but the dominant pathway probably depends on the scenario encountered. Studies using CD4$^-$CD8$^-$ double knockout mice showed that such mice could generate alloreactive T cells and that these animals were able to reject allogeneic skin grafts (6). Despite this finding, a recent report using separate CD4$^-$ and CD8$^-$ knockout mice has stressed an absolute requirement for CD4$^+$ cells in the initiation of allograft rejection (7).

It is well established that two signals are required for the induction of an optimal T cell response. TCR ligation of antigenic peptide/MHC complexes provides signal 1, while costimulation, most importantly through CD28 ligation by B7 molecules, provides signal 2 (8; reviewed in Refs. 9 and 10). Ligation of the TCR in the presence of costimulation leads to proliferation, while TCR ligation in the absence of costimulation produces either no response or a state of hyporesponsiveness. Recently, it has been demonstrated that CD28:B7 ligation serves to prolong cell survival and prevent apoptotic death by up-regulation of the Bcl-xL molecule (11). The B7 family of costimulatory molecules has been shown to be important in both the development of a T cell response and, more specifically, in allograft rejection. This has led to many efforts to prevent T cell activation using strategies that allow signal 1, but block signal 2. For example, B7 blockade with CTLA4Ig (12) has shown protection in several animal models of graft rejection (13–15). However, it appears that costimulation either by IL-2 or through CD28:B7 interactions is not an absolute requirement for the development of all T cell responses (16–19).

The 2C TCR is one of the most well-characterized TCRs. The CTL clone 2C was initially isolated from a BALB.B mouse as an allospecific T cell that recognized L$^d$ on the mastocytoma P815 (20). The binding affinity of the 2C TCR for its defined alloantigen p2C/L$^d$ has been measured (21), and the crystal structure of the receptor is now known (22). In addition to its primary Ag, peptide p2C bound to L$^d$, the 2C TCR also binds the Ags SIYRYYGL/K$^b$ (23), dEV-8/K$^b$ (24), and many of the V$\beta$8-specific superantigens

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(25). T cell development has been characterized in 2C TCR transgenic mice with the use of the clonotypic Ab 1B2 (26–28). We have now crossed 2C TCR mice onto a RAG-1$^{-/-}$ background (29) to produce animals with a uniform population of resting CTL precursor cells. These TCR/RAG$^{-/-}$ mice (hereafter referred to as TCR/RAG mice) contain 95% CD8$^+$1B2$^+$ peripheral T cells and lack B cells and T cells of any other specificity. This strain provides an opportunity to examine the requirements for CTL activation in a defined system with well-characterized Ags.

To investigate the ability of 2C TCR/RAG mice to recognize alloantigen in vivo, the DBA/2-derived P815 tumor line (30), against which CTL clone 2C was originally selected, was used in various transplantation experiments. The P815 model of tumor rejection has also been studied extensively, and recent work has used various transfectants of this cell line to evaluate the effect of the costimulation by B7 family molecules that are ligands for CTLA-4 and CD28 (31–33). Possible immune recognition and elimination of P815 by 2C TCR/RAG mice was of interest because this system would lack the three well-characterized sources of help for the development of an alloreactive CTL response; there are no CD4$^+$ Th cells for either direct or indirect help, and because P815 does not express B7, there is not a clear source of signal 2 for direct CTL stimulation. Despite these deficiencies, we show that large allogeneic tumor burdens are indeed rejected in a CD8-dependent manner in the absence of B7-mediated costimulation or help from CD4$^+$ T cells.

## Materials and Methods

### Mice

2C TCR transgenic mice (27) were crossed with RAG-1$^{-/-}$ mice (29) obtained from The Jackson Laboratory (Bar Harbor, ME). $F_1$ mice were backcrossed to RAG$^{-/-}$ to yield TCR/RAG$^{-/-}$ mice. Mice were maintained in barrier cages at the University of Illinois animal care facility. BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IA). Transgenic mice used in experiments were between 6 and 12 wk of age. Additional experiments were performed using 2C TCR transgenic mice crossed onto a RAG-2$^{-/-}$ (34) background. These animals as well as DBA/2 mice were bred and housed at the University of Chicago animal care facility.

### Cell lines and Abs

P815 is the mastocytoma-derived tumor line (30) against which T cell clone 2C was initially selected (20). EL-4 is a syngeneic (H-2$^b$) T lymphoma-derived cell line. T2-L$^d$ is a human lymphoblastoid derived line deficient in peptide transport and transfected with the L$^d$ gene (35). All cell lines were maintained in complete RPMI 1640 medium containing 5 mM HEPES, 10% FCS, 1.3 mM L-glutamine, 50 $\mu$M 2-ME, penicillin, and streptomycin. 1B2 is a mouse IgG1 mAb that is clonotypic for the 2C TCR. The Abs 53-6.7 (anti-CD8), RM4-5 (anti-CD4), 53.2.1 (anti-Thy-1.2), H1.2F3 (anti-CD69), 7D4 (anti-CD25), and 4F10 (anti-CTLA-4) were obtained from PharMingen (San Diego, CA). Ab 30-5-7, a mouse IgG2a anti-L$^d$ mAb, is sensitive for peptide-bound L$^d$ molecules (36). P198 is an immunogenic tum$^-$ variant of P815 that was isolated and grown as previously described (37).

### Peptides

The peptides SIYRYYGL (23), dEV-8 (24), MCMV, p2C (38), and QL9 (39) were synthesized on an Applied Biosystems 430A instrument (Foster City, CA) using standard F-moc chemistry at the University of Illinois Biotechnology Center (Urbana, IL) and were analyzed for purity by mass spectrometry and for concentration by quantitative amino acid analysis. Peptides were purified by reverse phase HPLC on a $C_{18}$ column eluting with a linear 5 to 60% acetonitrile gradient over 60 min in 0.1% trifluoroacetic acid.

### Proliferation assays

Standard proliferation assays were performed in triplicate round-bottom wells of 96-well plates over a 72-h period in complete RPMI medium. [$^3$H]thymidine at 1 $\mu$Ci/well was added during the last 18 h of the incubation period. Cells were collected and harvested on glass filters using a semiautomated cell harvester (Cambridge Technology, Inc., Watertown, MA). In some experiments, supernatant from rat splenocytes treated with Con A was added at a 10% volume to the assays. Con A supernatant was combined 2/1 with 20% $\alpha$-methylmanoside to neutralize free Con A.

### Cytotoxicity assays

Cytotoxicity was examined in standard $^{51}$Cr release assays. Briefly, target cells were labeled with $^{51}$Cr for 1 h at 37°C. Some $10^4$ target cells/well were incubated with various numbers of effector cells for 4 h. Supernatants were harvested and assayed for specific lysis according to the formula: percent specific lysis = (counts per minute experimental − counts per minute spontaneous)/(counts per minute maximal − counts per minute spontaneous). In all cases spontaneous lysis was <25% of maximal lysis.

### Tumorigenicity experiments

Tumor cells, generally $10^7$ for i.p. experiments and $10^6$ for s.c. experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the left flank of mice. For s.c. tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were killed when tumors reached diameters >25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as either the day of death or the point when moribund mice were killed. Observation of mice injected i.p. was continued through a 10-wk period.

### Flow cytometry

Flow cytometry was performed at the University of Illinois Flow Cytometry Facility using a Coulter Epics XL instrument. Thymi or spleens were removed from mice and passed through wire mesh, and RBC were lysed. Generally, ~$10^5$ cells/tube were incubated for 30 min with the appropriately labeled Ab in a volume of 50 $\mu$l PBS/1.0% BSA on ice. Cells were washed and resuspended in 400 $\mu$l before analysis.

### Treatment of mice with murine CTLA4IgG3 fusion protein (mCTLA4γ3)[3]

Purified mCTLA4γ3 (32) and control IgG3 were adjusted to 100 $\mu$g/ml in PBS. Mice received 1 ml of either of these reagents on days −1, 0, 1, 2, 4, 7, 11, 14, 17, 21, and 24. Tumor cells were implanted several hours following the second injection on day 0.

## Results

### Characterization of TCR/RAG mice

The progeny of TCR/RAG$^{-/-}$ $F_1$ × RAG$^{-/-}$ mice were examined for serum IgM levels and 1B2$^+$ PBL. Mice that exhibited no detectable IgM by ELISA or 1B2$^+$ cells by flow cytometry served as founders for a colony of 2C TCR/RAG mice (data not shown). Two-color flow cytometry analysis using anti-Thy 1.2, and the clonotypic Ab 1B2 revealed that essentially 100% (99.4 ± 0.7%) of the T cells from the spleens of these mice were 2C T cells (Fig. 1). This showed not only that the mice express the transgenic TCR, but it confirmed the RAG$^{-/-}$ background of these mice, as no endogenously rearranged TCR genes (i.e., Thy 1.2$^+$/1B2$^-$ cells) were observed. This contrasts with 2C TCR transgenic mice (hereafter referred to as TCR mice), in which only 88 ± 5% of the splenic T cells were 1B2$^+$ ($p < 0.001$).

T cells from spleens and thymi of mice at different ages were examined using 1B2 and anti-CD4 and anti-CD8 Abs. Three-color flow cytometric analysis of 2C T cells from the thymus revealed that TCR/RAG mice had more CD4$^+$/CD8$^+$ cells and fewer CD4$^-$/CD8$^-$ than did the TCR mice (Fig. 2*A*). Within the TCR/RAG thymus, 35 ± 12% of the 2C thymocytes were CD4$^+$CD8$^+$, while in the TCR thymus, only 19 ± 7% of the 2C thymocytes were CD4$^+$CD8$^+$ ($p < 0.01$). Conversely, the number of CD4$^-$CD8$^-$ cells was reduced (24 ± 9%) in TCR/RAG mice compared with that in TCR mice (41 ± 9%; $p < 0.005$). Thus,

[3] Abbreviations used in this paper: mCTLA4γ3, murine CTLA4IgG3 fusion protein; $^{low}$, low level; PEC, peritoneal exudative cell.

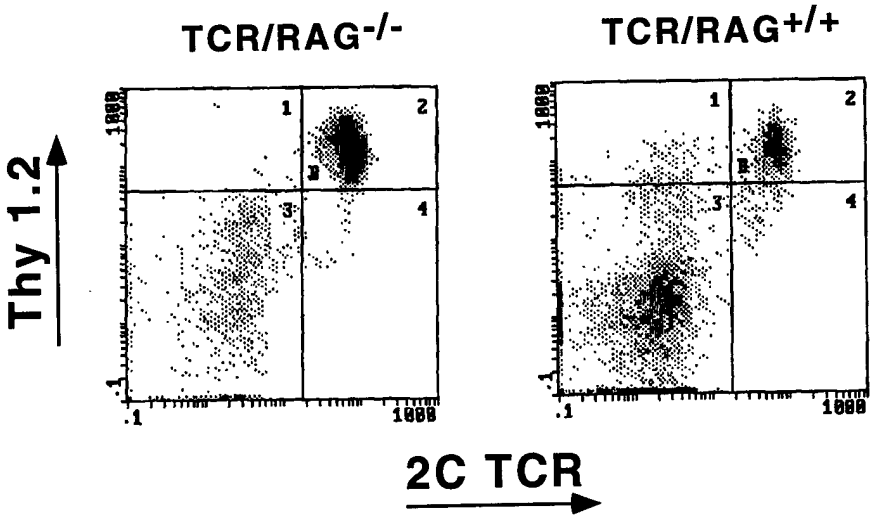


**FIGURE 1.** Characterization of T cell populations in TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 53.2.1 (anti-Thy 1.2) and 1B2 (2C TCR clonotypic).

TCR/RAG mice had about twice as many double-positive thymocytes and about half as many double-negative thymocytes as TCR mice. The significant number of $CD4^-CD8^-$ 2C T cells present in the spleens of TCR mice was also absent in the spleens of TCR/RAG mice (Fig. 2*B*). Within the spleens of TCR/RAG mice, 95 ± 2% of 2C T cells were $CD8^+$, while in spleens of TCR mice, only 65 ± 10% of 2C T cells were $CD8^+$, with the majority of the remainder being $CD4^-CD8^{low}$ or $CD4^-/CD8^-$ ($p < 0.0001$). Thus, in the TCR/RAG mice, ~95% of the entire T cell repertoire consists of $CD8^+$ CTL precursors, while in the TCR mice, only about 55% (45–70%) of all T cells are 2C $CD8^+$ CTL precursors. These results indicate that the prevention of endogenous TCR gene rearrangement during thymic development in the 2C TCR/RAG mouse leads almost exclusively to a $CD8^+$ CTL phenotype.

### *Ability of TCR/RAG to reject allogeneic tumors*

Since 2C TCR/RAG mice lack B cells or Th cells, they provide a unique opportunity to examine the requirements for the development of a CTL response. The allogeneic tumor line P815 lacks the B7 family of costimulatory molecules and thus provides a source of signal 1 (p2C/$L^d$-TCR ligation) in the absence of signal 2 (B7-CD28 ligation). To investigate whether TCR/RAG mice could reject an allogeneic tumor, P815 cells were injected either s.c. or i.p.

In the i.p. model, control $RAG^{-/-}$ animals ($n = 5$) injected with $10^7$ cells failed to reject the allogeneic tumors and died or became moribund and were killed within 3 wk (Fig. 3*A*). TCR/RAG mice ($n = 5$) injected with $10^7$ cells showed no signs of illness and survived the 10-wk period, with one exception (discussed later). Survival of TCR/RAG mice was significantly prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.005$). To examine the strength of the response, mice were injected with a 30-fold greater tumor load ($3 \times 10^8$ cells). Survival of these high tumor load animals ($n = 3$) was also prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.05$), but only one of the animals survived through 10 wk. without event. TCR/RAG mice injected with $10^7$ cells appeared to survive longer than those receiving $3 \times 10^8$ cells, although this result was not statistically significant ($p = 0.10$). In the s.c. tumor model, $RAG^{-/-}$ mice injected with $10^6$ P815 cells uniformly developed tumors that grew to a large size within 3 wk, while the TCR/RAG animals remained free of tumors and exhibited no illness throughout a 6-wk period (Fig. 3*B*). Tumor recurrence in these animals had not been observed through day 60 ($n = 3$). The fact that tumors were rejected by TCR/RAG mice but not by $RAG^{-/-}$ mice indicates that the transgenic $CD8^+$ CTL is necessary for the rejection.

To confirm the specificity of tumor rejection, the response of 2C TCR/RAG mice to s.c. injection of syngeneic EL4 tumor cells ($H\text{-}2^b$) was examined. EL4 tumors grew rapidly, while P815 tumors were uniformly rejected (Fig. 4). Coinjection of equivalent numbers of EL4 cells together with P815 cells delayed the growth of EL4 tumors by about 1 wk ($p < 0.0005$), but once established, growth rates for the EL4/P815 mix were similar to those for EL4 alone. Thus, while some bystander activity or peptide/MHC-independent killing may be responsible for slowing the initial growth of EL4, 2C CTLs are only able to cause the rejection of tumors bearing Ag of the correct specificity (i.e., p2C/$L^d$).

### *Tumor recrudescence is not attributable to tumor variant escape*

Two of the high tumor dose i.p. animals and one of the lower tumor dose i.p. animals exhibited tumor reoccurrence during the course of the 10-wk observation period. Analysis of one of these animals revealed a large ascites tumor yielding over $4 \times 10^8$ cells. Microscopic examination of a Wright's stained smear of this tumor demonstrated two distinct populations of cells: a smaller cell type appearing identical with P815 and a larger mononuclear granulocyte (data not shown). After culture for several days, a single cell population became dominant, which appeared identical with cultured P815 by microscopic examination. Flow cytometric analysis of the peritoneal lavage demonstrated that the recovered cells consisted of both $L^{d+}$ P815 tumor cells and a large number of activated peritoneal macrophages that were Thy $1.2^-$ and $CD25^+$ (Fig. 5*A*). Staining with the anti-$L^d$ Ab 30-5-7 demonstrated that the levels of $L^d$ were identical for the recovered tumor cells and cultured P815 (data not shown).

A CTL killing assay confirmed that both the recovered P815 tumor and cultured P815 were identical in their susceptibility to lysis by activated 2C CTLs (Fig. 5*B*). The responsiveness of the 2C T cells from these animals was not investigated, but it is possible that TCR/RAG mice have defects associated with memory responses, as has recently been observed in a TCR transgenic model of viral immunity (40). Thus, it appears that while tumor control is generally achieved and maintained, in at least some of the animals there is a failure to completely eradicate tumor cells, and late regrowth of tumor can occur. Moreover, this tumor recrudescence is not attributable to down-regulation of $L^d$ by the tumor cells or resistance to CTL-mediated killing.

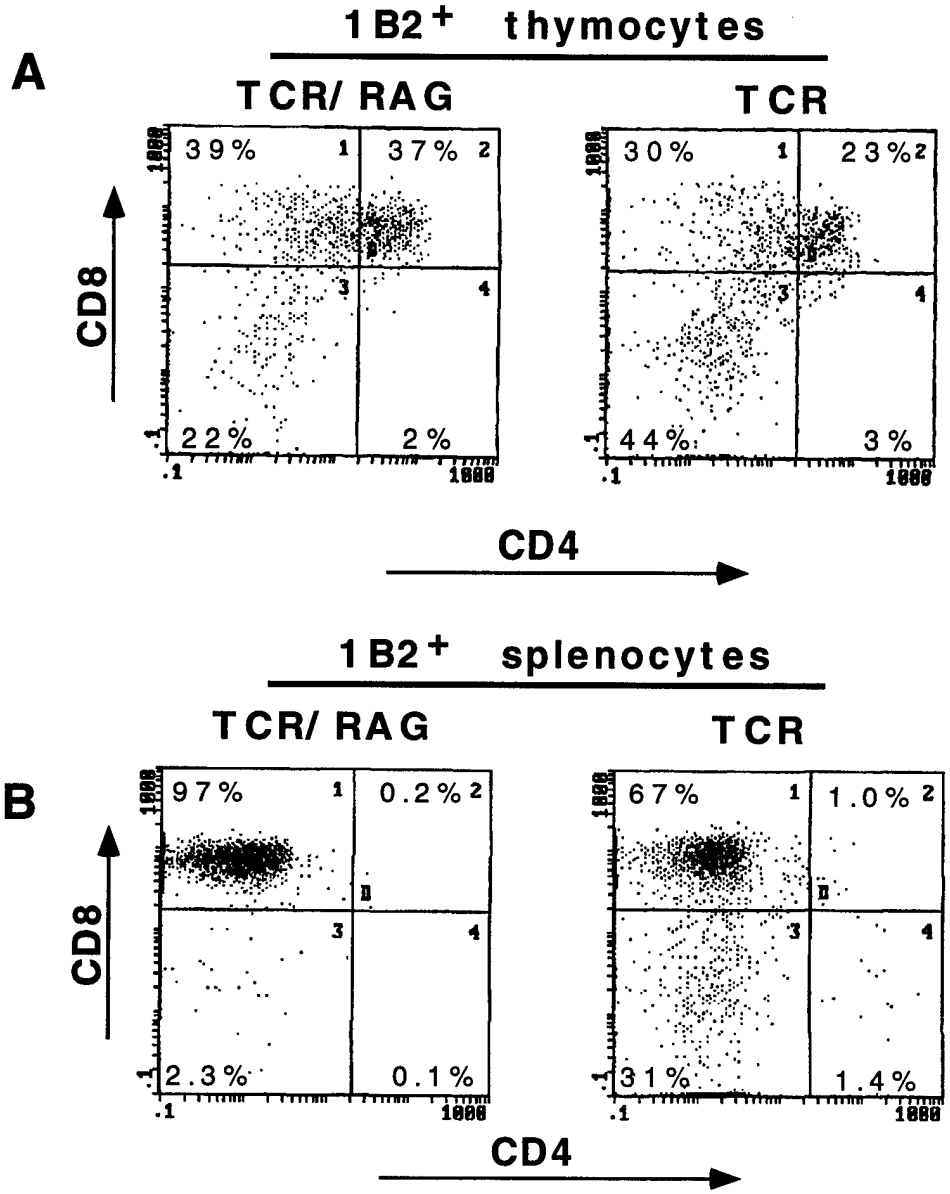


**FIGURE 2.** Comparison of 2C T cell populations from thymus and spleen of TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 1B2 (2C TCR clonotypic), 53-6.7 (anti-CD8), and RM4-5 (anti-CD4). *A*, The 1B2$^+$ population of thymocytes was analyzed for CD4 and CD8 expression by three-color staining. *B*, The 1B2$^+$ population of splenocytes was analyzed for CD4 and CD8 expression by three-color staining. Representative results for a single animal of each type are shown. Percentages given in the text reflect the average of six animals of each type.

### *Rejection of allogeneic tumors is B7 independent*

While P815 is a B7-negative tumor, it remained possible that host B7 molecules might be playing a role in costimulating 2C CTL development during a P815 tumor challenge. Such costimulation might be delivered either by host APCs acting in a bystander or *trans* manner or, alternatively, through the processing of an unknown tumor Ag through the CD8$^+$ indirect pathway. To assess whether a role for host B7 molecules exists in the rejection of B7-negative P815, mice were treated with mCTLA4γ3. The efficacy of the mCTLA4γ3 used in the experiments was confirmed by its ability to prevent rejection of the immunogenic P198 tumor in immunocompetent DBA/2 mice (Fig. 6*A*). The ability of B7 blockade to prevent rejection in this model has been taken as evidence for costimulation by host B7 in the rejection of B7-negative tumors (32). Treatment of 2C TCR/RAG mice with an identical course of mCTLA4γ3 or control IgG3 had no effect on the ability of these mice to reject s.c. P815 tumors (Fig. 6, *B* and *C*). RAG$^{-/-}$ mice injected with P815 uniformly grow large s.c. tumors within 3 wk (Fig. 6*B*), while both the mCTLA4γ3 and control treated groups are indistinguishable in their ability to reject these same tumors. Further, the same results were observed independent of the background of the mice (i.e., either RAG-1$^{-/-}$ (Fig. 6*B*) or RAG-2$^{-/-}$ (Fig. 6*C*)). These results illustrate that the observed tumor rejection is independent of both CD4-derived help and B7-mediated costimulation.

### *Characterization of the effector cell population during tumor rejection*

While it was clear that 2C CTLs were necessary for the tumor rejection observed in the TCR/RAG mice, it was not clear how

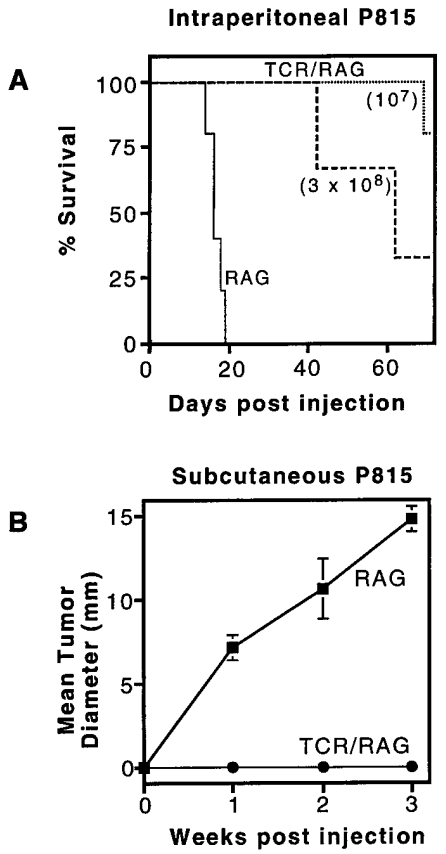


**FIGURE 3.** Tumor allograft rejection in TCR/RAG mice. *A*, TCR/RAG mice were injected i.p. with either $10^7$ P815 cells ($n$ = 5 mice) or 3 × $10^8$ P815 cells ($n$ = 3 mice). RAG mice received injections of $10^7$ P815 cells ($n$ = 5 mice). Animals were monitored as described in *Materials and Methods* for a period of 10 wk. *B*, TCR/RAG or RAG mice ($n$ = 3) were injected s.c. in the left flank with $10^6$ P815 cells in 100 μl and monitored weekly for tumor growth. The mean tumor diameter represents the average of two perpendicular measurements.

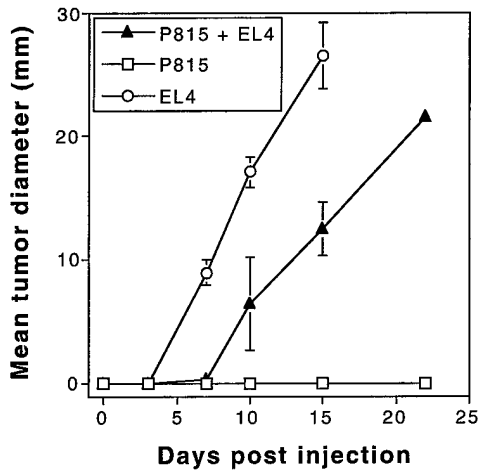


**FIGURE 4.** Tumoricidal activity in TCR/RAG mice is $L^d$ restricted. TCR/RAG mice were injected s.c. in the left flank with $10^6$ EL4 (H-$2^b$) or P815 (H-$2^d$) in 100 μl of PBS. Mice receiving coinjection of both cell types received $10^6$ of each in a single injection. Mice were monitored as described in *Materials and Methods*. The absence of error bars indicates a SD smaller than the size of the symbol.

these CTLs were accomplishing this task in the absence of any of the established sources of help for a CTL response. To explore the mechanism of tumor rejection, splenocytes and peritoneal exudative cells (PECs) from animals at various times following i.p. P815 injection were examined. Flow cytometric analysis of splenocytes exhibited only very low or background levels of the activation markers CD69, CD25, and CTLA-4 throughout the course of tumor rejection (data not shown). Further, only a low level (12%) of ex vivo cytolysis of P815 target cells was observed by splenocytes on day 4 (Fig. 7). This compares to cytolysis levels of typically >50% for cultured 2C effectors.

To assess whether 2C T cells were activated at the site of the tumor during the period of rejection, PECs were isolated from mice 4 days after i.p. injection of P815 tumor cells. When examined by flow cytometry, PECs showed blastic changes and stained positively for the T cell activation marker CD25, as well as for 2C TCR and CD8 (Fig. 8). In addition to $CD25^+$ blastic 2C T cells (Fig. 8, *B* and *C*), the peritoneal exudate consisted of a subset of smaller $CD25^-$, $CD8^+$ 2C T cells (Fig. 8*A*) as well as a more granular non-T cell population (Fig. 8*D*). The ability of PECs to lyse $L^d$-bearing target cells (T2-$L^d$) that were loaded with the peptide QL9 was examined. Ex vivo cytolytic activity of PECs varied from a low of 3% to a high of 40%, averaging 23% in the presence of the peptide QL9 (Table I). A variable amount of peptide-independent lytic activity averaging about one-third of the total lytic activity was observed in the unfractionated PECs. The peptide-dependent lytic activity (about two-thirds of the total activity) was inhibited to the peptide-independent level by addition of the anti-clonotypic Ab 1B2. This result suggests that the remaining peptide-independent lysis is not attributable to killing mediated through the 2C TCR (Table I). The response of TCR/RAG mice to an i.p. injection of BALB/c splenocytes (a B7-positive source of the p2C/$L^d$ Ag) was compared with that to an i.p. injection of P815. The results indicated no significant difference in either CD25 levels or lytic activity of 2C T cells isolated from the peritoneum (data not shown).

### *In vitro recognition of alloantigen*

It has previously been reported that purified $CD8^+$ 2C CTLs do not proliferate when cultured with P815 tumor cells unless exogenous IL-2 is added (41). We sought to confirm this result and determine whether there were any differences that might explain the rejection of P815 tumors by the TCR/RAG mouse. A comparison of the proliferative response to alloantigen in the form of either P815 tumor cells or BALB/c splenocytes (both $L^d$ positive) revealed a marked disparity depending on the nature of the APC (Fig. 9). Proliferation increased as the density of BALB/c APC increased, while proliferation decreased at higher P815 stimulator densities. In the case of both BALB/c and P815, addition of exogenous cytokines increased the proliferative responses. Depending upon the number of stimulator cells used in a single point assay, the ratio of proliferative responses to BALB/c vs P815 ranged from as high as 500:1 to as low as 2:1. When exogenous cytokines were present, this ratio varied from a high of 12:1 to a low of 1:1.5. Thus, it is evident that TCR/RAG splenocytes are indeed capable of proliferation in response to P815 tumor cells (21,000 cpm maximally), but at high densities of stimulators the response to BALB/c far exceeds the response to P815.

### *In vivo activation by self-MHC-restricted Ag*

To determine whether the 2C CTLs were capable of full activation in vivo, soluble antigenic peptides were injected i.p. on 2 consecutive days, and splenocytes were examined for activation on the third day. The peptides included MCMV, a $K^b$-restricted peptide that is not recognized by the 2C TCR; p2C and dEV-8, peptides that in complex with $K^b$ are recognized with low affinity ($K_a \cong$

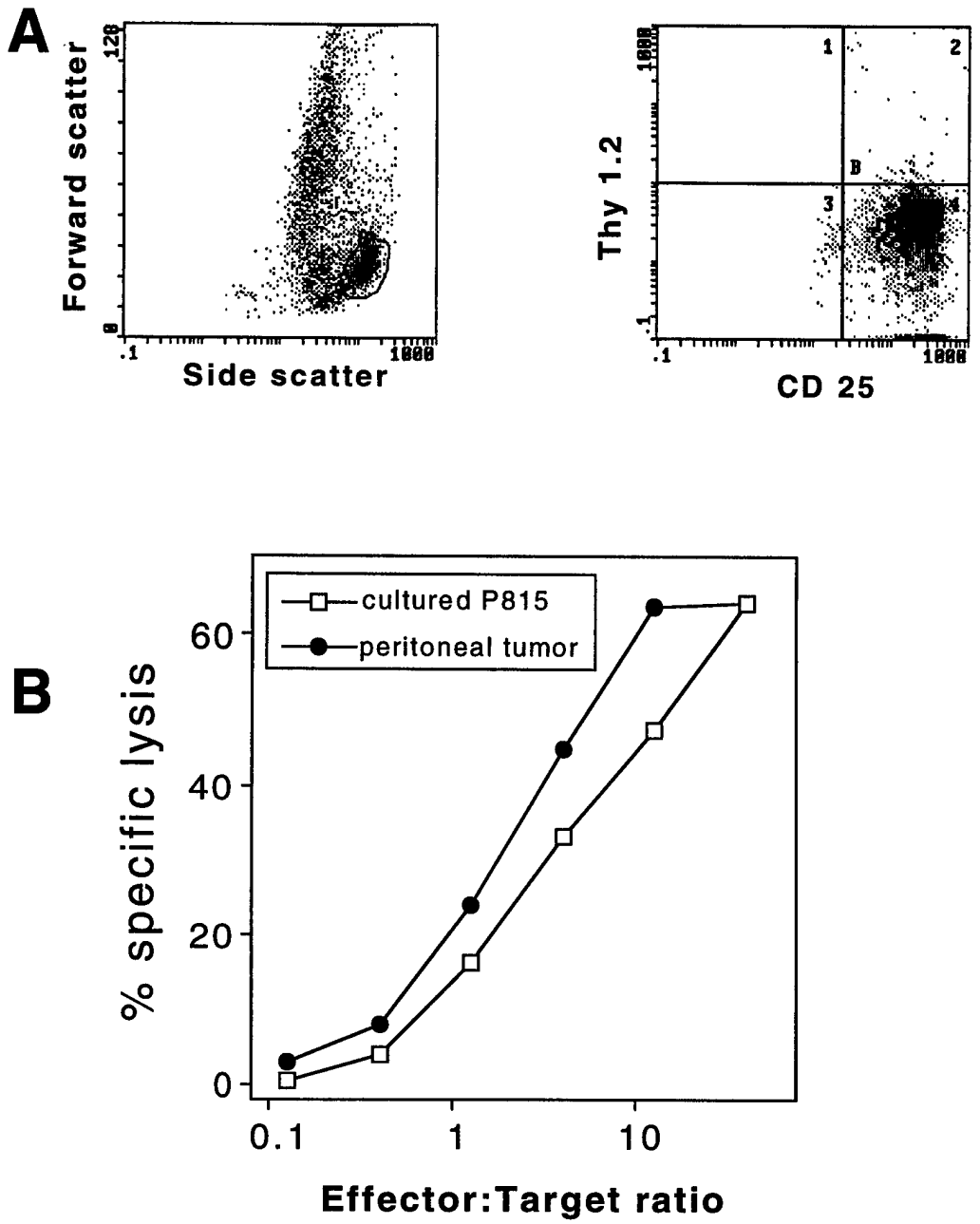


**FIGURE 5.** Characterization of peritoneal cell population in a TCR/RAG mouse with late tumor reoccurrence. Cells ($4.5 \times 10^8$) were harvested by peritoneal lavage of an ascites tumor from a moribund mouse injected 69 days earlier with $10^7$ P815 cells. *A*, Flow cytometric analysis of the cell populations isolated from the peritoneum of the same animal. The population of macrophages was gated and analyzed for expression of Thy 1.2 and CD25. *B*, Ascites tumor cells that had been in culture for 5 days and cultured P815 were assayed in a standard 4-h $^{51}Cr$ release killing assay for susceptibility to lysis by in vitro activated 2C CTLs.

$3 \times 10^3$ and $2 \times 10^5$ $M^{-1}$, respectively) by CTL 2C (21, 22); and SIYRYYGL, a synthetic peptide that was selected from a peptide library based on its ability to sensitize $K^b$-bearing cells to lysis by 2C (23). Only the SIYRYYGL peptide was able to vigorously activate T cells, as measured by expression of the activation marker CD69 24 h after injection (Fig. 10*A*). Essentially 100% of the $1B2^+$ T cells in the spleen become $CD69^+$ following exposure to this peptide.

To confirm that these activated cells were also capable of cytolysis, the ability of splenocytes from peptide-injected animals to lyse P815 target cells was examined. Again, only the SIYRYYGL peptide elicited splenocytes able to lyse $L^d$-bearing target cells ex vivo (Fig. 10*B*). Interestingly, injection of the peptide dEV-8, a putative positively selecting peptide for CTL 2C, also failed to produce detectable ex vivo cytolytic activity (Fig. 10*B*) or activation marker up-regulation (data not shown). Thus, 2C CTLs in the TCR/RAG could be fully activated after in vivo Ag challenge, but presumably only the higher affinity peptide/$K^b$ or TCR/peptide/$K^b$ interactions could achieve this activation. It is thought that the 2C TCR recognizes both p2C/$L^d$ and SIYRYYGL/$K^b$ with similar high affinity (23). These results confirm that the 2C CTL precursor cells in the TCR/RAG mice are indeed capable of full and complete activation in response to the appropriate peptide/MHC Ag. It suggests that the low levels of activation observed in the spleen after injection of P815 may be attributable to a lower load of Ag and localization of the Ag to the peritoneum.

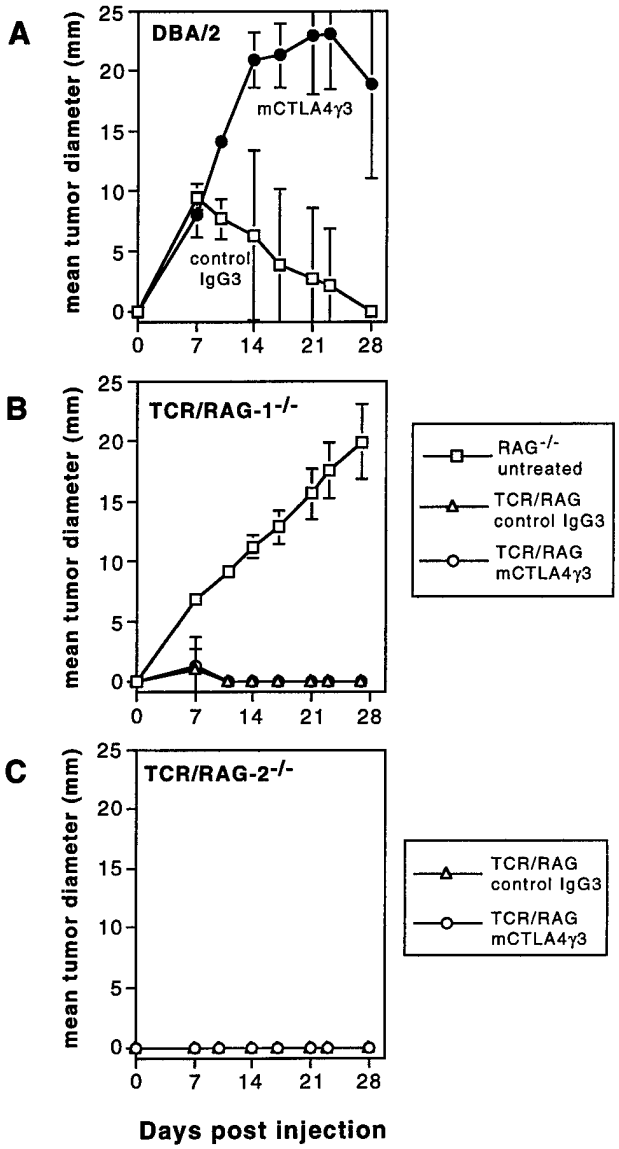


**FIGURE 6.** Role of B7 molecules in rejection of tumors. Mice were treated with a series of i.p. injections of either mCTLA4γ3 or control IgG3 beginning on day −1 as described in *Materials and Methods*. Tumor cells were injected on day 0 several hours after the second dose of mCTLA4γ3. *A*, DBA/2 mice were injected with $10^6$ P198 tumor cells in 100 μl of PBS in the left flank and monitored biweekly for tumor growth. *B* and *C*, 2C TCR/RAG mice on either a RAG-$1^{-/-}$ background (*B*) or a RAG-$2^{-/-}$ background (*C*) were injected with $10^6$ P815 tumor cells in 100 μl of PBS in the left flank and monitored biweekly. The mean tumor diameter represents the average of two perpendicular measurements. The absence of error bars indicates an SD smaller than the size of the symbol.

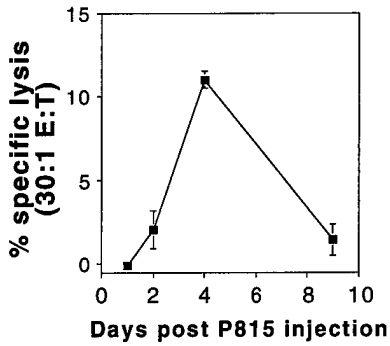


**FIGURE 7.** Kinetics of T cell activation in TCR/RAG mice during allorejection. Ex vivo cytolysis of splenocytes isolated on various days following tumor injection. Splenocytes were assayed against $^{51}Cr$-loaded P815 target cells at various E:T ratios. The lysis at a 30:1 E:T ratio is shown. Each point represents a single mouse. Error bars represent the SD of triplicate wells within the same assay. Fully activated 2C CTLs typically show >50% specific lysis at 30:1 E:T against P815 (data not shown).

## Discussion

The critical importance of costimulation in T cell-mediated responses has been repeatedly demonstrated within the last several years in numerous systems. Nevertheless, several situations have now been described where the most well-characterized costimulation signal, CD28:B7, is not an absolute requirement (16–19). We now present a system where allogeneic tumors are rejected in a B7-independent manner by a mouse that possesses only $CD8^+$ CTLs of a single specificity and that completely lacks B cells or Th cells.

Thymic development of 2C T cells appears to proceed along a slightly different kinetic course in TCR/RAG mice compared with TCR mice. Fewer thymocytes mature into $CD4^-CD8^-$ peripheral T cells in the TCR/RAG (5%) than in the TCR (45%). In contrast, there are proportionally twice as many $1B2^+CD4^+CD8^+$ thymocytes in the TCR/RAG thymus as in the TCR thymus. Mice transgenic for a TCR that was specific for MHC-I demonstrated increases in thymic emigrant numbers, but the increase was accompanied by a reduced size of the $CD4^+CD8^+$ thymocyte pool (42). This reduced double-positive pool is not observed when the transgenic 2C TCR is expressed on a RAG background. The explanation for why there are more mature $CD4^-CD8^-$ 2C T cells in the periphery of TCR mice than in TCR/RAG mice is not clear. The $CD8^+$ and $CD8^-$ subsets of 2C cells in the TCR animal are distinct and nonoverlapping and have been purified by others (43). In the TCR mouse, transient expression of endogenous TCR chains during thymic development might reduce the number of transgenic 2C receptors. A reduction in 2C TCR density may lead to altered signaling and a resultant loss of both CD4 and CD8 expression from 2C cells. Variations in the number of TCR per cell required for signaling various T cell functions has recently been described (25, 44).

The fact that 2C TCR/RAG mice are able to reject even large tumor burdens while their corresponding $RAG^{-/-}$ littermates succumb to the tumors indicates that the cell type responsible for this allorejection is the $CD8^+$ CTL bearing the 2C TCR. Our s.c. dose of $10^6$ P815 cells is 20-fold greater than the minimal tumorigenic dose used in many syngeneic animal models (31), while our i.p. tumor dose is 10-fold greater still. Because this animal lacks $CD4^+$ T cells or, in fact, T cells that might recognize any other Ag, the CTL response must be independent of CD4 help. What, then, is the source of help necessary for the rejection of these tumors? There are several possibilities. First, it may be that the rejection is made possible by the sheer number of CTL precursors and the high intrinsic affinity ($2 \times 10^6\ M^{-1}$) of the 2C TCR/p2C/$L^d$ interaction, which could abrogate the requirement for costimulation, as has been proposed previously (43). That is, since p2C is immunodominant for $L^d$, and P815 expresses high levels of $L^d$, the high epitope density and high TCR affinity may act to overcome the requirement for costimulation. A sufficient number of CTLs may secrete their own cytokines and become activated locally to a level that is capable of initiating the lytic response and controlling the tumor.

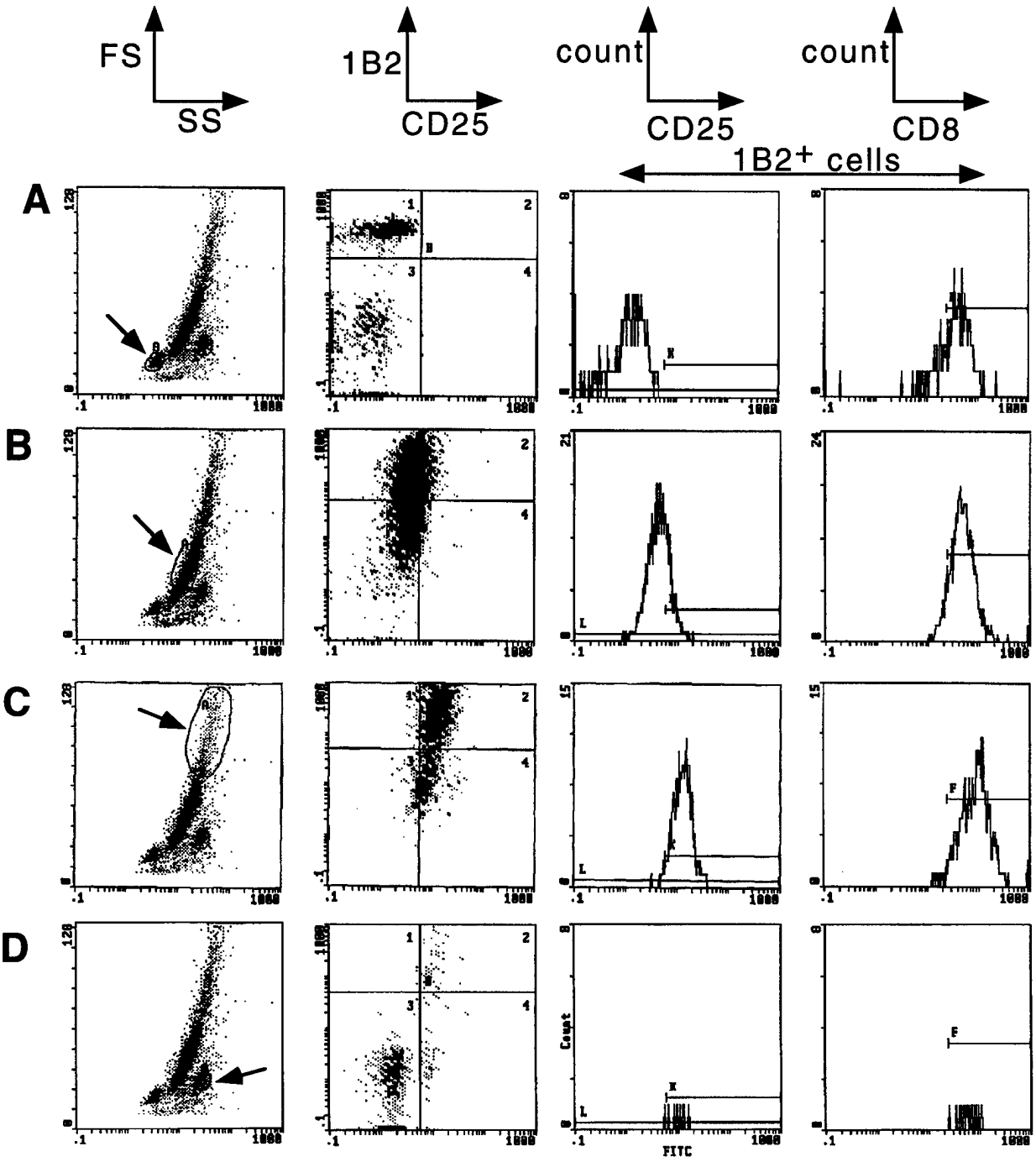


**FIGURE 8.** PEC populations at the time of tumor rejection. PECs were isolated by peritoneal lavage from mice injected with P815 tumor cells 4 days earlier. Distinct populations were gated upon (gate indicated by arrow) and analyzed for various T cell markers by flow cytometry. *A*, Smaller CD25$^-$ 2C CTL precursors. *B* and *C*, Larger, blastic CD25$^+$ 2C CTLs. *D*, Population of more granular, non-T cells. Results were representative of several mice analyzed.

A second explanation may involve an interplay between T cells and the innate immune system. It has been shown that macrophages and other cell types, such as NK cells, can participate in cytokine cascades that direct a T cell response toward either Th1 or Th2. Moreover, a requirement for NK cells in the induction of alloreactive human CTLs has been demonstrated (45). Such an interplay with the innate immune system operating in the local environment of the peritoneal cavity or an s.c. site might provide the necessary help needed for the development of CTL precursors into lytic CTLs. In Figure 9, the low level of proliferation in response to P815 is shifted nearly fourfold by the addition of exogenous cytokines. Thus, it is possible that other cell types in the local environment might be providing the requisite cytokines. For example, the IL-1$\beta$/IL-12/IFN-$\gamma$ cytokine cascade involves macrophages and NK cells (46). IL-12 secreted by macrophages or dendritic cells is known to be a potent inducer of Th1/Tc1 responses and is probably acting along with Ag to help promote CTL development.

A third possibility is that other costimulatory pathways could be replacing B7:CD28 in producing CTLs capable of tumor cell lysis. P815 is known to express intercellular adhesion molecule-1, which binds the LFA-1 Ag on T cells and can provide costimulatory

Table I. *Lysis of $^{51}$Cr-labeled T2-L$^d$ target cells by TCR/RAG PECs*[a]

| Mouse | % Lysis by PECs at 30:1 E:T (+QL9) | % Lysis by PECs at 30:1 E:T (No Peptide) | % Peptide Dependent | % 1B2 Inhibition (+QL9) |
|---|---|---|---|---|
| 1 | 33 | 20 | 40 | n.d. |
| 2 | 20 | 14 | 30 | n.d. |
| 3 | 40 | 4.6 | 89 | 91 |
| 4 | 33 | 0.8 | 97 | 83 |
| 5 | 2.8 | 0.4 | 86 | 89 |
| 6 | 8.3 | 3.0 | 64 | 54 |
| Average | 23 | 7.1 | 68 | |

[a] Effector cells were obtained by peritoneal lavage 4 days following i.p. injection of $10^7$ P815 cells. Assays were performed as described in *Materials and Methods*. Maximal 1B2 inhibition was assessed at a 1B2 concentration of 3–10 μg/ml. n.d. indicates that 1B2 inhibition was not examined in the indicated mouse.

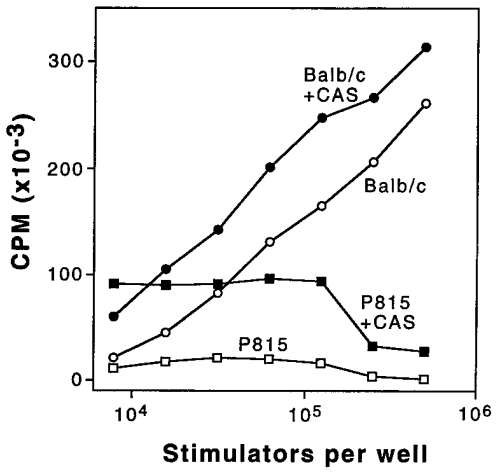


**FIGURE 9.** In vitro proliferation of 2C CTL precursors in response to alloantigen. Responder TCR/RAG splenocytes were cultured at 5 × $10^4$ cells/well with various numbers of allogeneic stimulator cells. Exogenous cytokine-rich supernatant from Con A-treated rat spleens (CAS) was added where indicated at a 10% concentration. Proliferation of stimulators or responders cultured alone yielded background levels <1500 cpm. The maximum proliferation in the presence of P815 without CAS was 21,000 cpm.

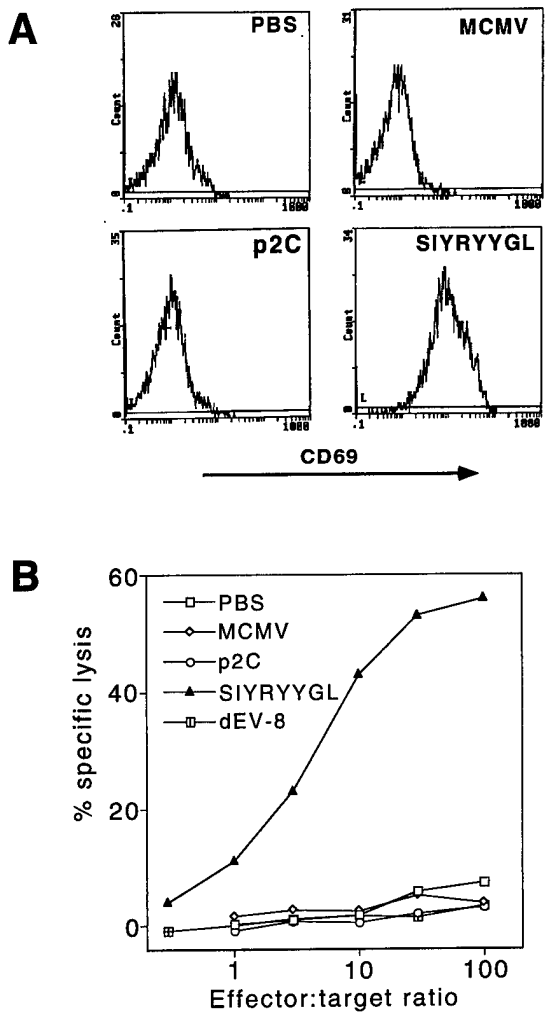


**FIGURE 10.** Peripheral activation of 2C T cells after injection of self-restricted peptide. *A*, Up-regulation of CD69 T cell activation marker on 1B2$^+$ T cells on day 3 following i.p. injection of antigenic peptide on days 1 and 2. Amounts of peptide injected for the figures shown are 70 nmol for MCMV and p2C and 10 nmol for SIYRYYGL. Injection of larger amounts of SIYRYYGL frequently caused death of the animals secondary to shock-like effects. Peptides were injected i.p. in 200 μl of PBS. *B*, Ex vivo cytolytic activity of splenocytes from mice injected with antigenic peptides in PBS. Spleens were harvested, RBCs were lysed, and splenocytes were assayed at various concentrations against $^{51}$Cr-labeled P815 target cells in a standard 4-h assay.

signals to T cells under certain conditions (47, 48). It has been demonstrated that naive T cells from CD28-deficient, OVA-specific Th cell transgenic mice can initiate, but not sustain, an immune response (49). This system differs from our own in that other endogenous nontransgenic T cells are present, and the transgenic T cells are of the CD4$^+$ helper phenotype. Yet another alternate costimulatory pathway that could be substituting for B7:CD28 involves the CD58:CD2 interaction, which has been shown to elicit production of IFN-γ by CD8$^+$ T cells (50).

Each of these possible mechanisms is consistent with the observation that 2C T cells from the site of the tumor at the time of rejection express CD25 and are able to lyse the appropriate tumor cells ex vivo (Fig. 8 and Table I). The fact that the rejection of P815 appears to be independent of B7 does not suggest that B7 molecules are unimportant in the development of a CD8$^+$ CTL response, only that they are not critical for rejection in this system. It may well be that the 2C TCR/RAG mouse could reject much larger tumor burdens if the tumor was transfected with B7. Similarly, B7-positive tumors might be rejected by mice with much lower CTL precursor frequencies.

The fact that several of the TCR/RAG animals eventually succumbed to tumors at a very late stage (Fig. 3*A*) indicates that while tumor control is generally achieved, 2C CTLs did not eliminate the entire tumor load in at least some cases. This result is reminiscent of the persistence of viremia in CD4-deficient or B cell-deficient mice (40, 51, 52), providing support for the idea that CD4$^+$ T cells are important not only for initial CTL priming, but also to help sustain the CTL response (40). A recent report using purified CD8$^+$ 2C T cells has suggested that the maintenance of CTL effector function is dependent on either Th cell-derived cytokines or costimulatory ligands distinct from B7–1 (53). The high number of activated macrophages isolated from the peritoneum (Fig. 5*A*) implies that a source exists for the IFN-γ necessary for the activation of these cells (54, 55). The nature of this source is unknown, although T cells or NK cells could be providing it. The observation that recovered tumor cells and cultured P815 exhibited equivalent susceptibility to killing suggests that the 2C T cells from these animals have become exhausted or peripherally tolerized. In this

regard, the 2C TCR/RAG mice should provide a useful system for investigating $CD8^+$ T cell memory and tolerance.

We did not observe high levels of activation in splenic T cells obtained from mice during the period when rejection was occurring (Fig. 7 and data not shown). It is clear, however, from the in vivo response to SIYRYYGL that the CTL precursors in the TCR/RAG are indeed capable of full activation (Fig. 10). The effector response in the peritoneum of tumor-rejecting mice is characterized by an infiltrate consisting of both 2C CTLs and nonspecific effector cells. It is likely that the effector cell kinetics vary somewhat in both peak strength and timing between various mice. The fact that the lytic activity of the unfractionated PECs is about one-third peptide independent and about two-thirds peptide dependent (Table I) suggests that the 2C T cells may have initiated a Th1-like local response, recruiting and activating macrophages, neutrophils, and NK cells, which may all attack tumor cells. While macrophage-mediated tumor killing is typically measured in an 18- to 24-h assay, at least some of the peptide-independent $^{51}Cr$ release by unfractionated PECs may be attributable to this cell population. This is consistent with a recent report examining the role of Ag receptor-negative cells in the effector response to an adenocarcinoma (56). Moreover, such a Th1-like response (57) initiated by $CD8^+$ CTLs would explain the delayed onset of EL4 tumors in mice injected with a mixture of EL4 and P815 (Fig. 4). However, the ultimate failure to reject these tumors provides additional evidence for the critical role of the 2C CTLs in the development and maintenance of an antitumor response.

It has recently been demonstrated that CD28 costimulation promotes production of the Th2-like cytokines IL-4 and IL-5 by both $CD4^+$ and $CD8^+$ T cells (58). It is conceivable that presentation of Ag by B7-negative P815 to 2C pCTL might result in less Th2/Tc2 differentiation, allowing a skewing of the T cell response toward the Th1/Tc1 phenotype. Alternatively, another possible explanation for the significant levels of peptide-independent lysis seen by PECs during the period of rejection could rest in the recent observation that Th2-type $CD8^+$ effectors show extensive peptide-independent killing of target cells (59) and also differ from Th1-type $CD8^+$ effectors in their levels of Fas-mediated cytolysis (60). The high numbers of activated macrophages in the animal with tumor recrudescence suggests that a perhaps ineffective Th1-type response may have predominated in this particular mouse. It remains to be determined which of the two pathways of development (Th1- or Th2-like) is followed by naive 2C T cells when challenged with P815 in vivo.

It was previously reported that purified $CD8^+$ 2C CTLs could not proliferate in response to P815 (41). To reconcile our observation that 2C T cells do proliferate weakly in response to P815 with these previous results, it is important to realize that the number of stimulator cells in the assay can have a profound impact on whether proliferation is observed. At the highest densities used in this earlier work ($2 \times 10^5$/well), we also observed similar low levels of proliferation. However, at lower stimulator cell densities, significant proliferation (up to 21,000 cpm) was detected even in the absence of exogenous cytokines (Fig. 9). This inverse effect of P815 cell density on the proliferative response of purified $CD8^+$ T cells was also described in a previous report (61). Moreover, it has recently been shown that the initial response to large doses of Ag does not require costimulation, but that costimulation plays a key role in prolonging the response (43). These findings are in keeping with the idea that costimulation up-regulates molecules that serve to protect against apoptotic death (11). In fact, the observation that tumors can reoccur in some mice suggests that the ability to mount a prolonged response may have been affected. We are currently examining this possibility.

Allograft rejection by TCR ($RAG^{+/+}$) mice has previously been examined (62), and it was found that female H-Y-specific TCR transgenic mice failed to reject male skin grafts. The authors suggested that this failure to reject might be attributed to a deficiency of $CD4^+$ Th cells secondary to skewing of the repertoire toward $CD8^+$ CTLs. In contrast, we found that the absence of $CD4^+$ cells does not prohibit rejection of allogeneic tumors. These results might be explained by differences in the type of allograft (i.e., skin- vs hemopoietic-derived tumor cells). In this respect, it will be of interest to examine the responses of 2C TCR/RAG mice to skin and cardiac allografts. Alternatively, the explanation may lie in differences in the overall avidity of the TCR/Ag interaction, taking into account both the high epitope density and the intrinsic TCR affinity of the 2C TCR/p2C/$L^d$ interaction and the lower avidity of the H-Y/TCR interaction. Recent work has demonstrated that 2C TCR mice ($RAG^{+/+}$) will vigorously reject cardiac allografts in a situation where all three sources of help for a CTL response are possible (63). The density and load of allograft cells may well be critical in controlling the extent to which CTL precursors either become activated or become peripherally tolerized. It was recently reported that adoptive transfer of 2C T cells (both $CD8^+$ and $CD8^-$) into $L^d$-bearing SCID mice (where $L^d$ is expressed on all nucleated cells) resulted in a tolerant state achieved through both activation-induced apoptosis and down-regulation of CD8 and the TCR (64). In contrast, when cardiac allografts were transplanted into 2C TCR transgenic mice ($RAG^{+/+}$) treated with rapamycin, T cells showed impaired intracellular signaling but unchanged surface levels of 1B2 and CD8 (63). Clearly, the density of $L^d$-expressing cells is much greater in an $L^d$ SCID mouse than in the case of cardiac allografts or $L^d$-bearing tumor cells.

In conclusion, we have described a system in which allorejection occurs in the absence of $CD4^+$ Th cells and independent of costimulation through CD28-B7 interactions. The cell responsible for this allorejection is an MHC class I-restricted, $CD8^+$ CTL of a single specificity acting in the absence of $CD4^+$ cells. Our results suggest that these $CD8^+$ effectors may be mediating their effects at least partly by the induction of a Th1-like local response to the allogeneic tumor cells. These findings highlight the flexibility of the immune response and raise additional questions about an absolute requirement for $CD4^+$ cells in allorejection (7). In addition, the 2C TCR/RAG mice provide a useful system to develop and test agents such as bispecific Abs and other immunomodulating agents that can effectively redirect and/or prolong the activity of a CTL response against target cells.

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# Targeting tumor cells with bispecific antibodies and T cells

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**Abstract**

It has been known for some time that mammalian immune systems are capable of eliminating large tumor burdens. Redirecting the immune response of a patient to an established tumor has now become the focus of various therapeutic strategies. In this report, two projects toward this goal are described. The first project involves the development of a transgenic mouse model for T cell directed therapeutics. These mice express specific T cell receptor α and β transgenes on a background in which the recombinational-activating-gene-1 (RAG) has been knocked out. The mice express cytotoxic T cells but not either T helper cells or B cells. Despite these deficiencies, the animals are capable of eliminating tumors that express the appropriate peptide/major histocompatibility complex ligand that is recognized by the αβ transgenic T cell receptor. Human tumors grow as transplants in these mice, thereby allowing various agents that redirect the endogenous T cells against human tumors to be tested. The second project involves a description of such agents: bispecific antibodies that simultaneously bind to an immune effector cell and a tumor cell. The bispecific antibody described here consists of folate attached to anti-T cell receptor antibodies, or their fragments. A single-chain Fv coupled with folate can redirect the lysis of human tumor cells that bear the high affinity folate receptor. Preliminary in vivo data showed that the folate/antibody conjugates were also capable of mediating rejection of the human tumor. This transgenic mouse model should now allow the evaluation and optimization of bispecific agents that can redirect a patient's own T cell response. © 1998 Elsevier Science B.V.

*Keywords:* Cytotoxic T Lymphocytes; Transgenic Mice; Bispecific Antibodies; Folate Receptor

## 1. Introduction

Most antibody-mediated targeting systems rely on the delivery of a linked, toxic compound in order to destroy the tumor. These approaches must overcome potential side effects associated with the toxic compound, either in the conjugate form or after release from the antibody. An alternative strategy, that has now been under investigation for the past 12 years, is to use the patients own T cells as the 'toxic' moiety. There are several potential advantages of using T cells in this capacity: (1) cytotoxic T lymphocytes (CTL) are present in large numbers in an individual; (2) each CTL has multiple mechanisms for killing a target cell, thereby limiting the outgrowth of tumor variants that might escape a single mechanism; (3) each CTL can kill multiple tumor targets; and (4) CTLs are a normal (i.e. self) component of an individual, and thus they are not recognized and cleared by the immune system, as might be the case with some toxins.

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One strategy to redirect the activity of T cells to a tumor has been the use of bispecific antibodies [1] that contain one antibody to a tumor antigen and another antibody to the T cell receptor complex (TCR). The bispecific antibody can mediate the lysis of the tumor cell by bridging CTL and tumor cell and by activating the CTL lytic machinery through the TCR complex. However, before a CTL can kill a tumor cell, it must be activated through simultaneous triggering of multiple cell surface molecules. There are now various strategies to accomplish such activation [2]. The goals of our work are to understand how a patients own CTLs might be most easily and effectively used, in conjunction with bispecific antibodies, as a therapeutic agent in cancer.

Two projects will be described in this report. The first involves the generation of a transgenic strain of mice (TCR/RAG) that will accept human tumor transplants, yet which contains endogenous CTLs. Thus, various drugs that can potentially be used for the activation and retargeting of CTLs to human tumors in vivo can be tested in these mice. Examples of the potency of tumor rejection by CTLs in these TCR/RAG mice will be shown. The second project involves the design and evaluation of the smallest bispecific antibodies yet produced, a conjugate of folate and an anti-TCR single-chain antibody (~30 kDa). These conjugates target the high affinity folate receptor (FR) present on most ovarian cancers and some brain tumors [3,4].

## 2. Materials and methods

### 2.1. Generation of transgenic mice

Mice that express αβ TCR trangenes from the CTL clone called 2C [5,6] were crossed with recombination activating gene-1 knockout mice (RAG−/−) [7]. Some T cells from the TCR transgenic mice express endogenous TCR and thus these mice contain T helper cells and they are capable of rejecting allogeneic and xenogeneic tumor transplants. In contrast, RAG−/− mice do not contain either B cells or T cells because they are not able to rearrange either Ig or TCR genes. The progeny of this cross and F1 backcrossed mice were examined for serum IgM and with the 2C TCR-specific mAb 1B2 [8] in order to develop a colony of TCR/RAG−/−mice. Serum IgM was monitored with an enzyme-linked immunoassay in which anti-kappa IgG coated on plates was used to capture serum Ig and bound IgM was detected with an HRP-labeled anti-mouse μ secondary antibody. To control for the total amount of serum added, various serum dilutions containing lysed red blood cells were analyzed for hemoglobin that was released, at an absorbance of 410 nm.

### 2.2. Tumor cell lines and transplantation

Tumor cell lines were used either as targets in cytotoxicity assays or in transplantation experiments with TCR/RAG mice. The following DBA/2 (H-$2^d$) derived tumor cell lines [9,10] were used as in vitro target cells: Mel, a murine erythroleukemia cell and F2-$MTX^r$A, a methotrexate resistant line selected for increased expression of FR by growth on low folic acid. The allogeneic mastocytoma line P815 (H-$2^d$), the syngeneic lymphoma EL-4 (H-$2^b$) and the human tumor cell lines SKOV-3, SKBR-3, BT474 (each erbB-$2^+$), and the $FR^+$ tumor KB [11] were transplanted into TCR/RAG mice and, in some cases, used as target cells in vitro.

Tumor cells, generally $10^7$ for intraperitoneal experiments and $10^6$ for subcutaneous experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the shaved back of mice. For subcutaneous tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were sacrificed when tumors reached diameters above 25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as either the day of death or the point when moribund mice were sacrificed. Observation of mice injected i.p. was continued through a ten week period.

### 2.3. Bispecific antibodies

The following monoclonal antibodies were used: 1B2, a mouse IgG1 specific for the TCR of CTL 2C [8] and KJ16, a rat IgG antibody specific for the Vβ8 region of the TCR [12]. KJ16 Fab fragments and KJ16 scFv were generated and purified as described

previously [13]. Briefly, scFv was refolded from inclusion bodies and monomeric scFv was purified by G-200 HPLC purification. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously [14,15]. Antibody conjugates were analyzed by mass spectrometry and absorption spectrophotometry.

### 2.4. In vitro CTL assays

The activity of CTL were examined in a cytotoxicity assay, in which tumor target cells were labeled with $^{51}Cr$ and effector cells were added with or without bispecific antibodies. Folate/antibody conjugates and cells were diluted in folate-free media and added to triplicate wells. Plates were incubated for 4 h and supernatants were removed for gamma counting. Cytotoxicity was determined by: % specific release=({experimental counts−spontaneous counts}/{maximal counts−spontaneous counts}×100), where spontaneous release is measured in the absence of CTLs and maximal release is measured with detergent lysis.

## 3. Results and discussion

Results of the two projects involving tumor cell targeting with bispecific antibodies and T cells will be described. The first project is designed to evaluate the potency of CTL-mediated tumor rejection, by using a transgenic mice that express CTLs but not T helper cells or B cells. The second project describes an approach to redirecting the activity of CTL, using a novel form of bispecific antibody.

### 3.1. Transgenic mouse model for CTL-mediated tumor rejection

In the normal host response to tumor antigens, there is a complex interaction among immune cells that leads to the elimination of the tumor cell. This interaction is known to involve T helper cells, antigen presenting cells (e.g. B cells and macrophages), and CTLs. We wished to determine if CTLs alone are capable of eliminating tumor cells and, if so, what strategies might be optimum for redirecting their activity exclusively to a tumor burden. Furthermore, we wished to develop an animal model that would allow human tumors to be successfully transplanted but would also contain endogenous CTL for in vivo targeting studies (unlike nude and SCID mice which lack T cells).

To approach these questions, a strain of mice (TCR/RAG) was produced that contains only CTLs of a single TCR type and specificity (and which does not recognize any known human tumor antigens) (2) (Fig. 1). The TCR/RAG mice were produced by breeding a transgenic mouse that expresses the α and β chains from CTL clone 2C [5,6] and RAG-1 knockout mice [7]. The phenotype of adult TCR/RAG animals was examined in order to determine if they indeed lacked all B cells and T helper cells (i.e. CD4+ cells). Screening of offspring of different ages, from matings of TCR tg mice with RAG−/− mice, was performed by flow cytometry of spleen cells. These results revealed that essentially all of the splenic T cells are $CD8^+$ (i.e. CTLs) (Table 1) and that they all express the same TCR as identified with an anti-TCR antibody, 1B2 [8]. TCR/RAG mice exhibited, on average, slightly reduced spleen and thymus sizes compared to normal mice or TCR transgenic mice (Table 1 and data not shown). The mice also lack any B cells, as evidenced by the lack of serum IgM (Fig. 2).

Despite the presence of only CTLs in the TCR/

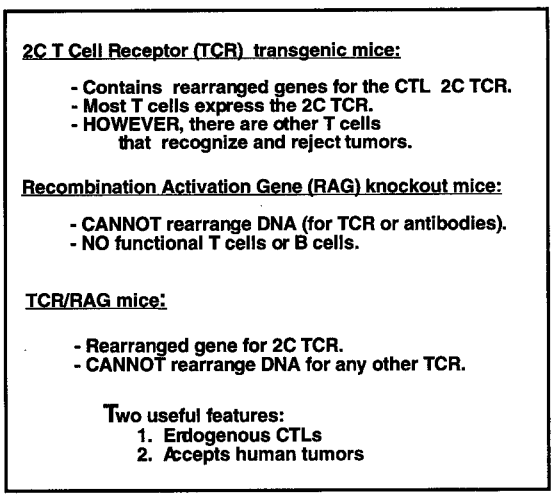


Fig. 1. Features of the 2C CTL TCR transgenic mice, RAG-1 knockout mice, and the TCR/$RAG^{-/-}$ progeny.

Table 1
Flow cytometric analysis of splenic lymphocyte populations in TCR/RAG-1$^{-/-}$ mice

| Strain/Age | Sex | Percent of T cells[a] | | | Spleen wt (mg) | Cell count ($\times 10^7$) |
|---|---|---|---|---|---|---|
| | | CD4+8− | CD4+8+ | CD4−8+ | | |
| C57/B6 | | | | | | |
| 5 mo | F | 66 | 0 | 33 | 80 | 5 |
| 5 mo | F | 55 | 0 | 45 | 82 | 6 |
| 5 mo | M | 63 | 0 | 37 | 228 | |
| 2C TCR | | | | | | |
| 5 mo | F | 31 | 0 | 72 | 107 | |
| TCRRAG−/− | | | | | | |
| 1 mo | F | 1 | 1 | 98 | 36 | .6 |
| 1 mo | M | 4 | 2 | 94 | 80 | 1.7 |
| 2 mo | F | 1 | 1 | 98 | 92 | 3.2 |
| 2 mo | F | 1 | 1 | 98 | 54 | 2.2 |
| 2 mo | M | 2 | 1 | 97 | 52 | 1.1 |
| 2 mo | M | 1 | 1 | 98 | 42 | |
| 3 mo | M | 1 | 1 | 98 | 113 | |
| 3 mo | M | 5 | 3 | 93 | 98 | |
| 4 mo | M | 2 | 0 | 98 | 47 | 1.9 |
| 4 mo | M | 1 | 0 | 99 | | |
| 5 mo | M | 1 | 2 | 96 | 75 | |
| 5 mo | M | 1 | 0 | 99 | 144 | |
| 5 mo | F | 0 | 2 | 98 | 91 | |
| 5 mo | F | 0 | 1 | 99 | 58 | |
| 6 mo | M | 5 | 0 | 95 | 48 | |
| 6 mo | F | 1 | 0 | 99 | 186 | |
| TCRRAG−/− | median | 1 | 1 | 98 | 75 | |

[a]Total T cells was calculated as (CD4+8−)+(CD4+8+)+(CD4−8+).

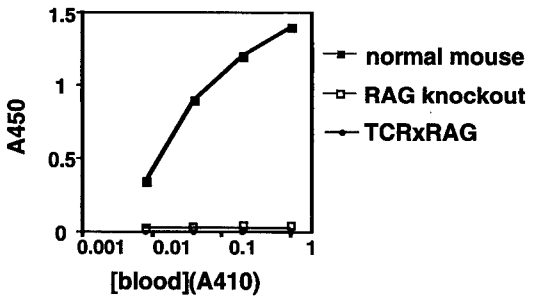


Fig. 2. ELISA of serum IgM from a normal C57Bl/6 mouse, a RAG-1$^{-/-}$ mouse, and a TCR/RAG$^{-/-}$ mouse. Blood was lysed by hypotonic shock and diluted for analysis in 96-well plates. Absorption at 410 nm of these samples was monitored immediately, prior to completion of the ELISA, as a measure of the amount of hemoglobin (i.e. directly proportional to the amount of blood in the sample). After these measurements were made, a standard ELISA for serum IgM was performed using anti-IgM specific antibodies, as described in Section 2.

RAG mice, CTLs could be activated both in vitro and in vivo with appropriate stimulating antigens. The transgenic TCR recognizes a peptide p2C (LSPFPFDL) bound to the class I MHC $L^d$ [16] or a peptide SIYRYYGL bound to the class I MHC $K^b$ [17]. Spleen cells from TCR/RAG mice injected with the SIYRYYGL peptide or spleen cells that were cultured with either SIYRYYGL/$K^b$ or p2C/$L^d$ proliferated and were shown to be activated by various criteria: expression of IL-2 receptors, expression of the CD69 antigen, and their ability to kill tumor cells that bear these antigens (Ref. Manning et al. submitted for publication).

In addition, the TCR/RAG mice were fully capable of eliminating tumor cells that bear the appropriate peptide/MHC ligand recognized by the tg TCR. The p2C/$L^d$ complex is expressed by the DBA/2 tumor P815, but not by various other tumor cell lines. To investigate whether the TCR/RAG could reject this allogeneic tumor load, P815 cells

were injected either subcutaneously or intraperitoneally. The TCR/RAG animals injected with $10^7$ cells showed no signs of illness and with one exception, survived without event (Fig. 3). Control RAG−/− animals failed to reject the allogeneic tumors and died or became moribund within three weeks. To examine the strength of the response, mice were injected with a 30-fold greater tumor load ($3\times10^8$ P815 cells). Two of three high tumor load animals survived through the six week experimental period without event, and one succumbed to its tumor late in the course at day 42. The observation that tumors are rejected by the TCR/RAG animals and not rejected in the RAG mice indicates that the transgenic $CD8^+$ CTL is the cell type responsible for the rejection. In addition, TCR/RAG mice failed to reject other mouse tumors such as EL4, that do not express the appropriate ligand recognized by the transgenic CTL (Fig. 4). This result further shows that peptide/$L^d$-specific CTL of the TCR/RAG mice are required for rejection.

Finally, various human tumor cell lines were transplanted into TCR/RAG mice in order to determine if they developed tumors and thus could be used as models for drugs that target CTLs to tumors. The erbB-$2^+$ tumors SKOV3 and BT474 (but not SKBR-3) and the high affinity $FR^+$ tumor line KB all grew as subcutaneous tumors (Fig. 4 and data not shown). Because human tumor cells are not killed by activated 2C CTLs, we expected that the KB tumor would grow in TCR/RAG mice even when endogenous T cells were activated by administration of the SIYRYYGL peptide. This expectation was met, as the KB tumor grew equally well in peptide-treated and untreated mice (Fig. 4). Thus, the KB tumor line can serve as a model for targeting of activated CTL, using folate/anti-TCR conjugates described below.

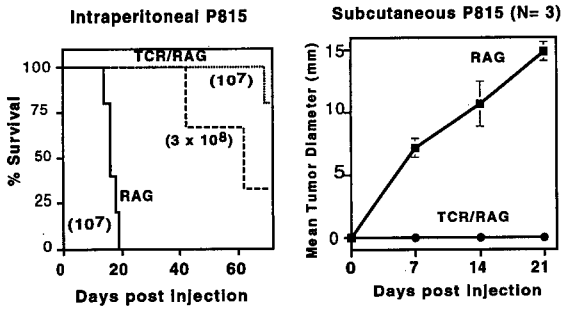


Fig. 3. Tumor allograft rejection in TCR/RAG mice. In the panel on the left, TCR/$RAG^{-/-}$ mice were injected i.p. with either $10^7$ P815 cells ($n$=5 mice) or $3\times10^8$ P815 cells ($n$=3 mice). RAG-$1^{-/-}$ mice received injections of $10^7$ P815 cells ($n$=5 mice). Animals were monitored as described in Section 2 for a period of ten weeks. In the panel on the right, RAG-$1^{-/-}$ or TCR/$RAG^{-/-}$ mice ($n$=3) were injected s.c. in the shaved back with $10^6$ P815 cells in 100 μl and monitored weekly for tumor growth. Mean tumor diameter represents the average of two perpendicular measurements.

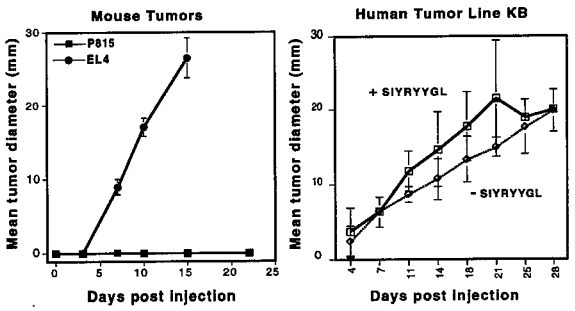


Fig. 4. TCR/$RAG^{-/-}$ mice do not reject tumors that lack the appropriate peptide/MHC ligand. In the panel on the left, TCR/RAG mice were injected s.c. with $10^6$ EL4 (H-$2^b$) or P815 (H-$2^d$) in the shaved back in 100 μl of PBS. In the panel on the right, TCR/$RAG^{-/-}$ mice were injected s.c. with $3\times10^6$ human KB tumor cells. Mice received either 10 nmoles peptide SIYRYYGL (+SIYRYYGL) or saline (−SIYRYYGL) 2 days prior to tumor transplantation. $N$=3 for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

### 3.2. Characterization of bispecific folate/antibody conjugates

Bispecific antibodies that bind to a tumor antigen and the TCR redirect CTLs to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the FR, is expressed on most ovarian carcinomas. Recently, we showed that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein [18].

The size of these conjugates can be reduced to the smallest bispecific agent yet described (30 kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody called KJ16 (Fig. 5). Folate was attached through a carboxyl group to scFv

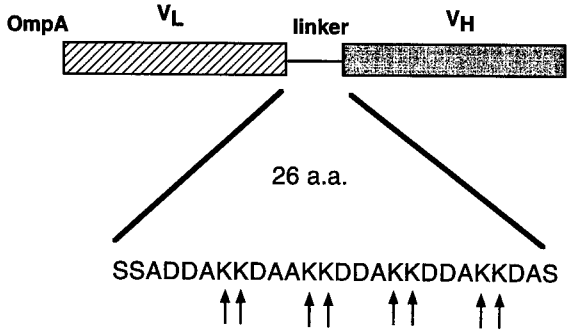


Fig. 5. Diagram of the single-chain Fv from the anti-T cell receptor antibody KJ16. The 26 amino acid linker that contains multiple lysine residues for carbodiimide coupling of folate is shown.

amines using a carbodiimide reaction. The scFv/folate conjugates were as effective as intact IgG/folate conjugates in mediating lysis of $FR^+$ tumor cells by CTL (Fig. 6) [19]. The optimal folate density was in the range of 5 to 15 folate molecules per scFv or IgG molecule, which yielded $EC_{50}$ values of approximately 40 pM (1.2 ng $ml^{-1}$ for scFv). The scFv/folate conjugates could also efficiently target tumor cells in vitro even in the presence of free folic acid at concentrations that are normally found in serum [19].

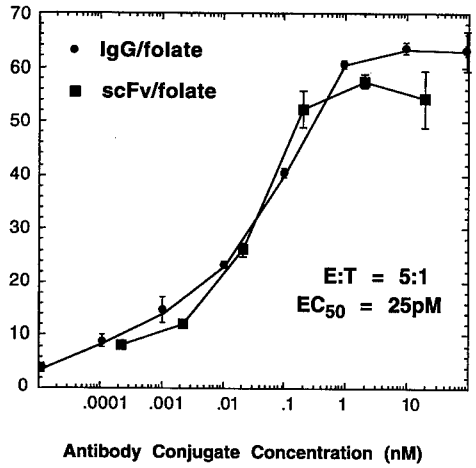


Fig. 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 μM EDC, 100:1 molar ratio of folate:antibody) yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using $^{51}$Cr-labeled F2-MTX$^r$A cells and CTL clone 2C.

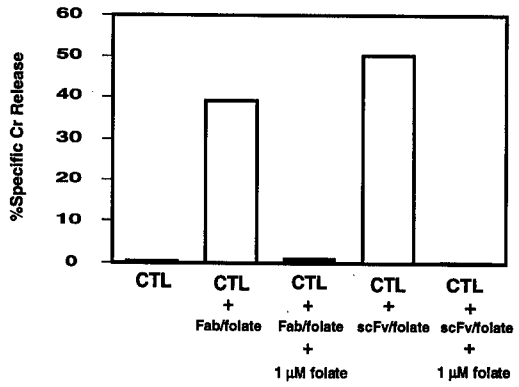


Fig. 7. Folate/antibody conjugates mediate lysis of $FR^+$ human tumor cell line KB by CTLs. Activated CTL from TCR/RAG mice were added to $^{51}$Cr-labeled KB cells in the presence of the indicated agents. KJ16 Fab/folate and KJ16 scFv/folate preparations were each assayed at ~10 μg $ml^{-1}$. Free folate was added at 1 μM.

In vitro-activated CTLs from the TCR/RAG mice described above were able to kill the human FR+ tumor line KB in the presence of folate/scFv and folate/Fab conjugates (Fig. 7). This redirected lysis was completely inhibited by excess free folate and thus the effect is mediated through the high affinity FR.

Finally, a preliminary study has shown that a Fab/folate conjugate was capable of mediating the rejection of KB tumor cells in TCR/RAG mice that had been injected with SIYRYYGL peptide to activate CTL (Fig. 8). In this experiment, mice were co-injected with 3 million KB cells and 20 μg of the folate/Fab conjugate subcutaneously. Studies to further evaluate the in vivo targeting efficiency of the folate/scFv and folate/Fab conjugates will focus on several issues: (1) methods of in vivo CTL-activation; (2) route and timing of antibody delivery; and (3) optimization of the effective concentration and forms of the conjugates.

## 4. Conclusion

Using a transgenic animal model, we have demonstrated that CTL alone are potent mediators of tumor

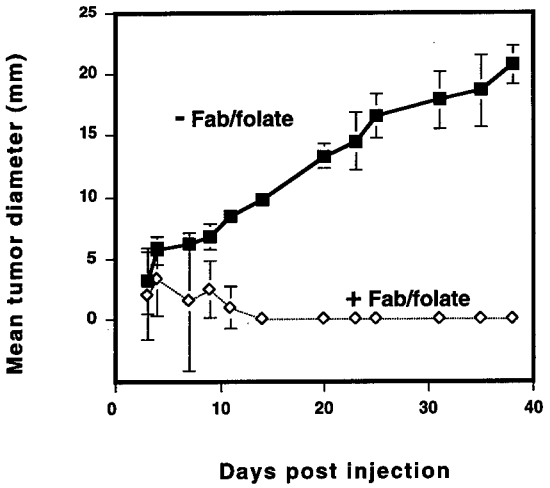


Fig. 8. Fab/folate conjugates mediate the rejection of KB tumor cells in TCR/RAG mice. TCR/RAG mice were injected s.c. with $3\times10^6$ human KB tumor cells in the presence (+) or absence (−) of 20 μg Fab KJ16/folate. Mice received 10 nmoles peptide SIYRYYGL, two days prior to tumor transplantation. $N=3$ for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

rejection. This finding suggests that it will be possible to focus strategies on the in vivo activation and retargeting of CTL to specific tumor cell antigens. In addition, the TCR/RAG mice can accept human tumor xenografts and thus they should serve as a useful model for the testing of bispecific agents that target human tumors.

In an effort to generate smaller and more potent bispecific agents, we have explored the use of folate/anti-TCR conjugates for targeting the high affinity FR, a tumor associated antigen present on most ovarian tumors. Folate directly coupled to a single chain $V_LV_H$ antibody yielded a potent tumor targeting agent in cytotoxicity assays with CTLs. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors, in reduced immunogenicity, and in eliminating Fc-mediated side effects.

## Acknowledgements

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# TARGETING T CELLS AGAINST BRAIN TUMORS WITH A BISPECIFIC LIGAND-ANTIBODY CONJUGATE

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**High-affinity receptors expressed on the surface of some tumors can be exploited by chemically conjugating the ligand for the receptor and an antibody against immune effector cells, thus redirecting their cytolytic potential against the tumor. Ovarian carcinomas and some brain tumors express the high-affinity folate receptor (FR). In this report, a transgenic mouse model that generates endogenously arising choroid plexus tumors was used to show that folate/anti-T-cell receptor antibody conjugates can direct infiltration of T cells into solid brain tumor masses. An engineered single-chain Fv form of the anti-T-cell receptor antibody KJ16 was conjugated with folate, to produce a bispecific agent that was substantially smaller than most previously characterized bispecific antibodies. Folate conjugation to the antibody increased T-cell infiltration into the tumors by 10- to 20-fold, and significantly prolonged survival of the mice. *Int. J. Cancer* 76:761–766, 1998.**


Tumors use a variety of mechanisms to avoid being eliminated by the immune system. Some tumors actively suppress immune function by secreting inhibitory cytokines or by killing T cells through Fas/FasL interaction. Other tumors escape recognition by cells of the immune system. For example, tumors may fail to present peptide antigens in complex with a product of the major histocompatibility complex (MHC) that are essential for recognition by T cells. Tumor cells may also be deficient in co-stimulatory ligands and adhesion molecules that facilitate recognition and T-cell activation. Finally, the early development of tumors may allow the immune system to become tolerant of potential surface antigens on tumors.

One potential way to overcome escape due to failures of recognition is to redirect immune cells against tumor cells with bispecific antibodies. Bispecific antibodies can be constructed to recognize 2 separate antigens, one on the tumor surface and the other on the surface of an immune effector such as a cytotoxic T cell. Many tumor cells have potential target antigens that are tumor specific or quantitatively more abundant on tumor cells than normal cells (tumor associated). Previous work has demonstrated the *in vitro* and *in vivo* effectiveness of bispecific antibodies against a variety of experimental tumors. Several clinical trials have been conducted with first-generation bispecific agents with results that are sufficiently promising to warrant further study of this strategy (Canevari *et al.,* 1995).

Despite considerable progress in the design of bispecific antibodies, significant obstacles to their effective clinical use remain. One of the problems associated with the bispecific antibody approach has been the difficulty in identifying animal models that can mimic human cancers. By analogy with human use, one would like a system that could evaluate how an endogenously arising tumor could be controlled by treatment with bispecific agents that redirect the activity of endogenous T cells. To date, all pre-clinical bispecific antibody studies have used transplanted tumors in either syngeneic rodent systems or xenogeneic systems in which human tumors and lymphocytes are transplanted in immunodeficient mice. This report shows that a new class of bispecific antibody agents can be used in a transgenic, endogenous tumor model to study T-cell-mediated therapies.

The tumor antigen targeted in this study was the high-affinity folate receptor (FR), which has been identified on ovarian carcinomas and most choroid plexus tumors and ependymomas (Mantovani *et al.,* 1994; Weitman *et al.,* 1992). The high-affinity FRs ($K_D$ approx. 1 nM) differ from the ubiquitous lower affinity reduced folate carriers ($K_D$ approx. 100 μM) that are largely responsible for normal folate uptake (Westerhof *et al.,* 1991). High-affinity FRs were originally identified as tumor-associated antigens using monoclonal antibodies that reacted with ovarian tumor cell lines (Coney *et al.,* 1991). The presence of FR on ovarian tumors has led to its use as a target for various forms of therapy, including bispecific antibodies (Mezzanzanica *et al.,* 1991).

The nanomolar affinity of folate for FR suggested that attachment of folate directly to an anti-T-cell receptor (TCR) antibody might be a rapid method to generate bispecific antibodies that efficiently target FR-positive tumor cells for lysis by activated T cells. In cytolytic assays, these folate/antibody conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (Kranz *et al.,* 1995). To reduce the size of the bispecific agent, we have produced folate conjugates of the single-chain Fv of the anti-Vβ8 antibody KJ16 (scFv KJ16), and the folate/scFv conjugates were as effective as the folate/IgG conjugates in cytotoxicity assays against FR-positive cells (Cho *et al.,* 1997). The 30 kDa folate/scFv conjugate is to our knowledge the smallest bispecific antibody yet reported.

To develop an animal model for testing the folate/bispecific agents, we chose SV11 mice that are transgenic for the SV40 large T antigen gene with the SV40 enhancer (Van Dyke *et al.,* 1985). SV11 mice develop choroid plexus tumors with 100% penetrance and in a well-defined time period (age of mortality averages about 100 days). SV40-induced choroid plexus tumors express FR with properties that are very similar to the human FR, including a $K_D$ of 1 nM (Patrick *et al.,* 1997). In addition, flow cytometry and immunohistochemistry of FR on tumor cells indicated that virtually all the viable cells are FR positive (data not shown).

## MATERIAL AND METHODS

### *Purification of scFv-KJ16*

scFv-KJ16 was solubilized from *E. coli* inclusion bodies and refolded by dilution into 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $CaCl_2$, 1 mM EDTA, 5 mM phenylmethylsulfonylchloride, as described previously (Cho *et al.,* 1995). After concentration by tangential flow, the scFv preparation was dialyzed into 20 mM Tris, pH 8.0, and purified by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) eluting with 20 mM Tris, pH 8.0, 1 M NaCl. Peak fractions were pooled, concentrated, and dialyzed into PBS if used *in vivo* or 0.1 M

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MOPS, pH 7.5, if used for folate conjugation. The purity and activity of the KJ16 scFv preparations were assessed using a competition flow cytometry assay as described previously (Cho *et al.,* 1995). scFv-KJ16 purified in this manner had an affinity for TCR as reported previously (approx. 120 nM) and was approx. 95% monomeric as determined by gel filtration (G200).

*Preparation of folate/scFv-KJ16 conjugates*

Folate scFv conjugates were prepared and characterized as described previously (Cho *et al.,* 1997). Briefly, folate and the crosslinker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce, Rockford, IL) were dissolved in DMSO at a 2.5 molar excess of EDC. After 30 min at room temperature in the dark, a 100-fold molar excess of the activated folate was added to the scFv-KJ16 previously dialyzed into 0.1 M MOPS. After 1 hr at room temperature, the sample was applied directly to a G-25 column pre-equilibrated in 0.1 M MOPS, pH 7.5. Fractions were characterized by absorbance at 280 and 363 nm, and peak fractions were pooled, dialyzed into PBS and filter sterilized. The biological activity of each preparation of the folate/scFv-KJ16 conjugate was tested in cytotoxicity assays before use *in vivo.* Folate/scFv-KJ16 conjugate preparations averaged 10 folate molecules per antibody molecule.

*Animals*

The SV11 strain was obtained from Dr. T. Van Dyke (University of North Carolina, Chapel Hill, NC). The strain is maintained heterozygous for SV40 large T antigen by mating transgene-positive males with C57BL/6J females and determining genotype of offspring by PCR for large T antigen. Transgene-positive males and females have the same survival curves.

In vivo *infiltration of T cells assessed by immunohistochemistry of CD3*

Control mice received 2 injections of PBS or staphylococcal enterotoxin B (SEB, Toxin Technologies, Miami, FL) (50 μg) followed by PBS. Experimental mice were injected with SEB and scFv-KJ16 without folate (10 μg, i.v., 18 hr after SEB), or SEB and folate/scFv-KJ16 conjugate (10 μg, i.v., 18 hr after SEB) and sacrificed after 1 or 2 days, at 93–95 days of age. Mice receiving SEB and IgG KJ16 or folate/IgG-KJ16 (10 μg) were sacrificed after 2 days. Tissues were fixed by perfusion with acetic acid/zinc/formalin (Newcomer Supply, Middleton, WI); the brains were then blocked into 2 mm thick pieces and paraffin embedded the next day by the University of Illinois College of Veterinary Medicine Histopathology Laboratory. Six blocks per slide were mounted together, allowing estimation of the total number of infiltrating T cells in a tumor. Three micron serial sections were deparaffinized, rehydrated to PBS, blocked with normal goat serum and incubated overnight at 4°C with primary antibody. The primary antibody was rabbit anti-CD3 (Dako, Carpinteria, CA), followed by biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA), avidin-biotin-HRP complex (Vector Elite ABC) and nickel-cobalt-enhanced diaminobenzidine (DAB; Pierce) for detection. Slides were counterstained with methyl green, dehydrated and coverslipped. Tumor areas were determined using NIH Image at 25×. Individual T cells were counted at 100× in 6 planes per brain; the total number of T cells in 6 planes ranged from 1 (a PBS-treated mouse) to 2,343 (a folate/scFv-KJ16 treated mouse). The number of mice in each condition was as follows: PBS (3), SEB (2), scFv-KJ16 at 24 hr (2), folate/scFv-KJ16 at 24 hr (4), scFv-KJ16 at 48 hr (4), folate/scFv-KJ16 at 48 hr (4), IgG-KJ16 at 48 hr (1) and folate/scFv-KJ16 at 48 hr (2).

In vivo *treatment to test survival benefit*

Experimental mice were placed on a low-folate diet at age 70 days to reduce serum folate levels. Commercial rodent diet contains high folate concentrations, and a reduced folate diet brings serum folate levels down to levels observed in humans (Mathias *et al.,* 1996). We measured serum folate levels in C57BL/6 mice fed a normal diet and a reduced folate diet and observed a reduction from 236 ± 12 nM to 56 ± 14 nM within a week, followed by relatively stable low levels (35 ± 3 nM at 4 weeks). In control experiments with n = 8 we found that neither the maintenance on low-folate diet nor the stress of repeated restraint and injection of PBS affected survival (data not shown). Baseline body weight of mice was obtained at 84 days. With previous cohorts of mice we correlated the loss of body weight due to cachexia and age of death to provide an objective criterion for morbidity. Mice died within 2–3 days of reaching 75% of baseline body weight, so this criterion was used to determine time of sacrifice; mice were also sacrificed if they showed clear neurological symptoms such as vestibular problems (from 4th ventricle tumors) or ataxia and lethargy.

Antibody-treated mice were injected i.p. with 100 μg SEB on day 84, followed 18 hr later by i.p. injection of either folate/scFv-KJ16 (25 μg in PBS, n = 8) or scFv-KJ16 (25 μg, n = 8). Mice received 3 additional i.p. injections of antibody (25 μg) at 4 day intervals. A single injection of SEB was employed to avoid SEB-induced anergy (Sabapathy *et al.,* 1994). Control mice (n = 9) were untreated. Mice were weighed and monitored daily for neurological signs.

*Statistical analysis*

Infiltration data were analyzed by analysis of variance using SAS JMP software. Survival data were analyzed by Kaplan-Meier estimates and the log-rank test, using SAS JMP.

## RESULTS

To determine whether the folate moiety of the bispecific conjugate could specifically target T cells against SV11 tumors, mice were injected with either scFv-KJ16 or folate/scFv-KJ16 preparations. T cells were activated 1 day before the injection of the bispecific agent by injecting the superantigen SEB, which activates the Vβ8 population of T cells recognized by KJ16. Mice were sacrificed 24 or 48 hr after antibody injection, and the number of T cells that infiltrated the tumor was evaluated by immunohistochemistry of CD3. In the absence of SEB or bispecific antibody, very few T cells were observed (as few as 1 or 2 per brain). SEB alone produced low levels of infiltration (Fig. 1*a*). Similarly, scFv-KJ16 without folate-conjugated antibody produced very low levels of T-cell infiltration (Fig. 1*b*). In contrast, treatment with the folate/scFv-KJ16 conjugate caused a substantial infiltration of T cells into the tumors (Fig. 1*c*). Quantification of T-cell density in the tumors confirmed that folate significantly increased T-cell infiltration both 24 and 48 hr following treatment ($p < 0.0001$, folate/scFv-KJ16 *vs.* scFv-KJ16, Fig. 2). The IgG form of folate/KJ16 produced a moderate level of T-cell infiltration at 48 hr but less than the single-chain form of the conjugate at the same time point (Fig. 2, $p = 0.05$).

Having demonstrated that the folate-conjugated antibody targeted T cells to the tumor, we then asked whether the folate/single-chain antibody conjugate was capable of redirecting a sufficient number of T cells to have an impact on survival. Minor modifications of the treatment protocol were made to optimize chances of improving survival. The animals were put on a low-folate diet to reduce circulating serum levels of folate that could compete with the bispecific agent, and multiple injections of a higher dose of folate/scFv-KJ16 were used.

Our colony of SV11 mice has an essentially identical survival curve to the original description of the strain over 30 generations ago, with survival times of 100–105 days for various cohorts. To estimate when to begin treatment, we characterized the histological development of tumors in untreated animals. Consistent with an earlier description (Van Dyke *et al.,* 1987), a small number of hyperplastic foci usually begin to appear between 70 and 80 days of age, and then tumors become increasingly anaplastic and grow approximately exponentially over the next 3–4 weeks (Fig. 3).

Based on this characterization of tumor development, mice were treated beginning at day 84 with SEB and either scFv-KJ16 or

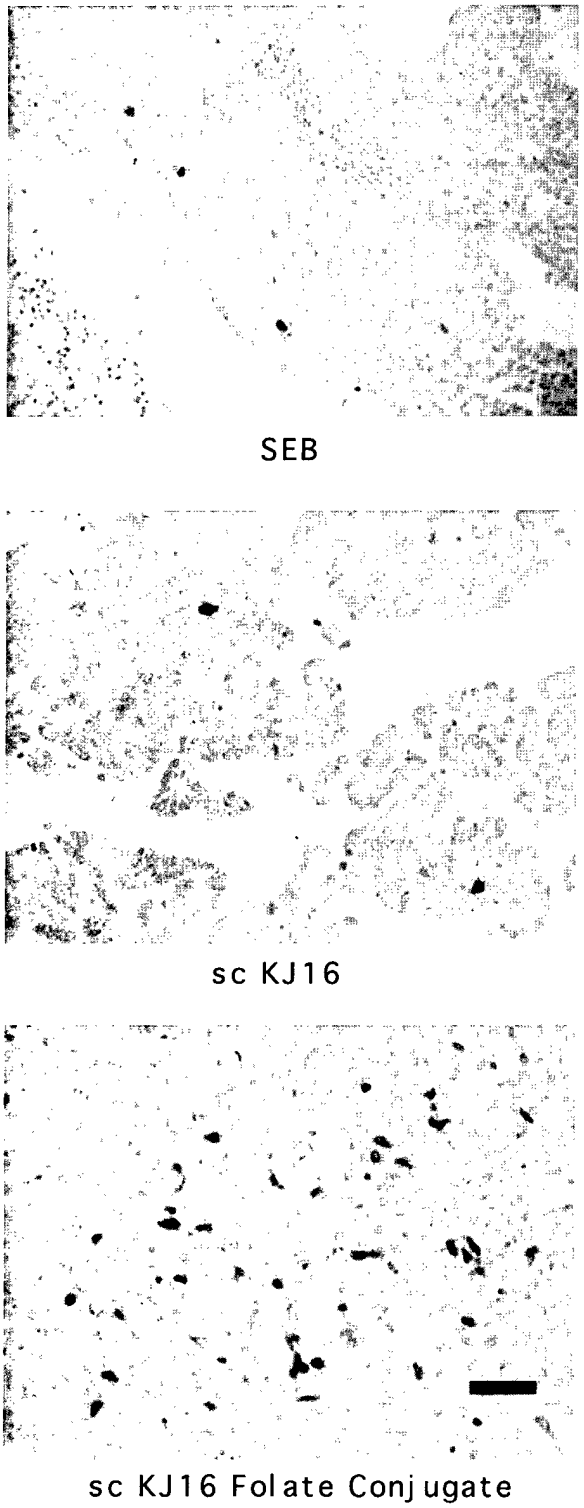


FIGURE 1 – Infiltration of choroid plexus tumor by T cells following treatment with folate/anti-TCR bispecific antibody. (*a*) Immunohistochemistry of T-cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.). Tumor cells are counterstained with methyl green. (*b*) Immunohistochemistry of T-cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.) and scFv-KJ16 antibody (10 μg i.v.) reveals minimal T-cell infiltration when folate is not conjugated to the antibody. (*c*) Folate/scFv-KJ16. Immunohistochemistry of CD3 following treatment with SEB (50 μg i.p.) and folate/scFv-KJ16 antibody (10 μg i.v.). Extensive infiltration of darkly staining T cells is observed. Scale bar = 50 μm.

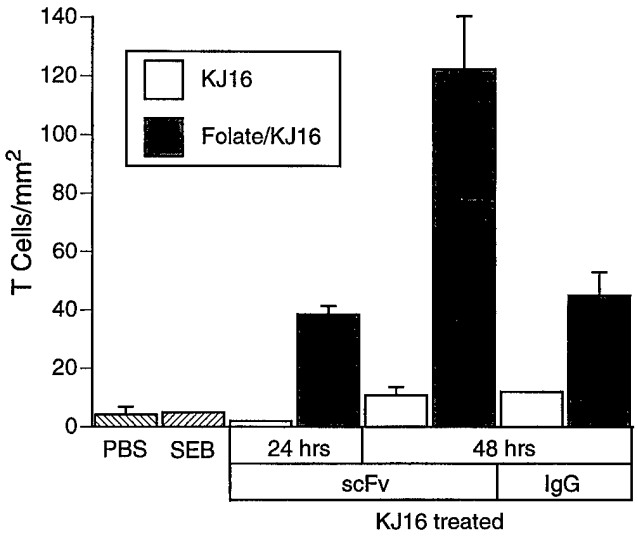


FIGURE 2 – Quantitation of T cell density in tumors following treatment with various anti-TCR antibody preparations. Controls were injected with PBS vehicle or superantigen SEB. Experimental mice were injected with SEB followed by 1 of 2 forms of the anti-Vβ8 TCR antibody KJ16, either single-chain Fv (scFv) or IgG. Each of the 2 forms of KJ16 was either conjugated with folate or non-conjugated. Mice receiving the scFv antibodies were sacrificed 24 or 48 hr following antibody treatment, and mice receiving IgG antibodies were sacrificed 48 hr following antibody treatment. Conjugation of the antibody with folate increased the infiltration of T cells into the tumor in all cases, with the highest density of T cells observed 48 hr following the scFv form of the folate conjugate.

folate/scFv-KJ16. A single dose of SEB was followed 18 hr later by an injection of antibody. Three additional injections of antibody were given over the next 12 days. Treatment with SEB and scFv-KJ16 resulted in a significant prolongation of survival compared with untreated controls ($p < 0.05$). However, treatment with SEB and folate/scFv-KJ16 resulted in a further significant enhancement of survival ($p < 0.05$ compared with scFv-KJ16, Fig. 4). Survival times were 100 days for controls, 112 days for scFv-KJ16 treated mice and 120 days for folate/scFv-KJ16 treated mice.

## DISCUSSION

We have demonstrated that endogenous T cells can be redirected to infiltrate and attack brain tumors. The action of T cells was sufficient to roughly double the survival time following the initial development of tumors in SV11 mice (*i.e.,* approx. 20 days from day 80 to 100 for untreated mice compared with approx. 40 days from day 80 to 120 for treated mice). Previous reports have demonstrated tumor infiltrating lymphocytes in established solid tumors after bispecific antibody treatment (Thibault *et al.,* 1996), but few have demonstrated a reduction in solid tumor growth or prolongation of survival (Kroesen *et al.,* 1995). The present findings are noteworthy given the difficulties posed by the blood-brain barrier and the relative immune privilege of the brain. The transgenic nature of the model makes it additionally challenging to treat; because of the expression of SV40 large T antigen in every choroid plexus cell, each is a potential new focus of tumor development. Thus, even if the existing tumors were completely eradicated, new tumors would presumably arise. It may therefore be necessary to develop a full memory response in T cells to cure tumors in this particular model.

Activation of T cells by SEB appeared to have a beneficial effect on survival, as mice treated with SEB/unconjugated scFv-KJ16

lived significantly longer than control mice. This finding is consistent with earlier reports that activation of T cells by SEB or an anti-CD3 antibody such as 145-2C11 can generate anti-tumor activity in mice (Newell *et al.,* 1991; Penna *et al.,* 1994). In a separate trial, we treated SV11 mice with 145-2C11 and produced a survival benefit similar to that in mice treated with SEB/unconjugated scFv-KJ16 in the present experiment (data not shown). We do not know why in both of these cases there was anti-tumor activity from these treatments in the absence of substantial T-cell infiltration, at least at early time points after the treatment (Fig. 1*a* and data not shown for 145-2C11). It may be relevant that for the present survival study that animals received twice the dose of SEB as the T-cell infiltration study. However, in earlier *in vivo* studies of SEB alone against murine tumors, doses ranging from 50 to 250 μg/mouse produced similar anti-tumor activity (Newell *et al.,* 1991; Penna *et al.,* 1994).

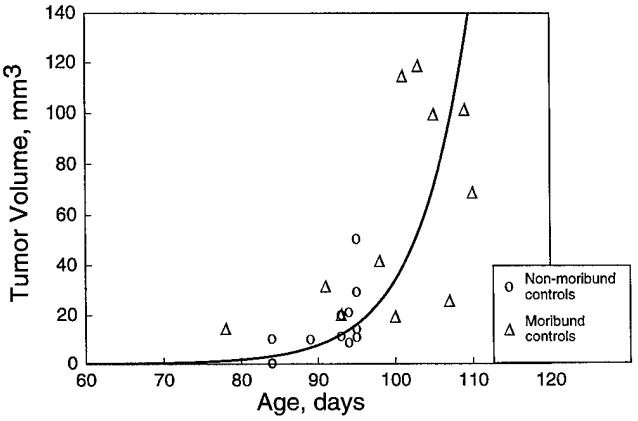


FIGURE 3 – Development of tumors in SV11 mice. Mice were sacrificed at various ages, either while still healthy (non-moribund, circles) or moribund by neurological or body weight criteria (triangles). Tumor volume was calculated from tumor area, determined with NIH Image from evenly spaced sections throughout the brain. Tumors develop rapidly after approximately day 80.

In the present study, we did not test the effect of the bispecific conjugate in mice that did not have T cells activated by SEB, but in another murine model (a transplant model of an FR-positive tumor), activation of T cells greatly enhanced the effect of the same antibody/conjugate (data not shown). Similarly, Penna *et al.* (1994) observed less effect of a bispecific antibody against melanoma cells when no *in vivo* activation with SEB was employed.

Our results emphasize the importance of both ligand-based targeting of tumors and the use of the smaller scFv form for systemic treatment of solid tumors. The folate conjugate of the scFv form of KJ16 elicited a substantial increase in T-cell infiltration and resulted in enhanced survival that was significantly greater than the scFv without folate. For example, there was an approx. 20-fold increase in T-cell number at 24 hr after treatment with folate/scFv-KJ16 and an approx. 10-fold increase in T-cell number at 48 hr after treatment compared with scFv-KJ16 without folate. The single-chain form of the antibody also appeared to be important, as the folate/scFv-KJ16 produced an approximately 3-fold higher number of T cells compared with the IgG form of the folate/KJ16 conjugate at 48 hr. However, we did not conduct a pharmacokinetic analysis of the 2 forms of antibody/conjugate, and the difference between single-chain and IgG forms may be a difference in time course. In previous comparisons of IgG and single-chain antibodies, the single-chain antibody has also provided better access of the antibody to T cells or better tumor penetration from systemic circulation (Yokota *et al.,* 1992). These measurements provide a useful guide to the possible relationship between the extent of T-cell infiltration into a tumor and the benefit regarding survival. In the present experiments, the animals investigated for T-cell infiltration were not on a low-folate diet, whereas the animals in the survival experiment were on a low-folate diet, so the actual number of T cells in the latter case may have been higher than we calculated. Extensions of these studies should allow us to

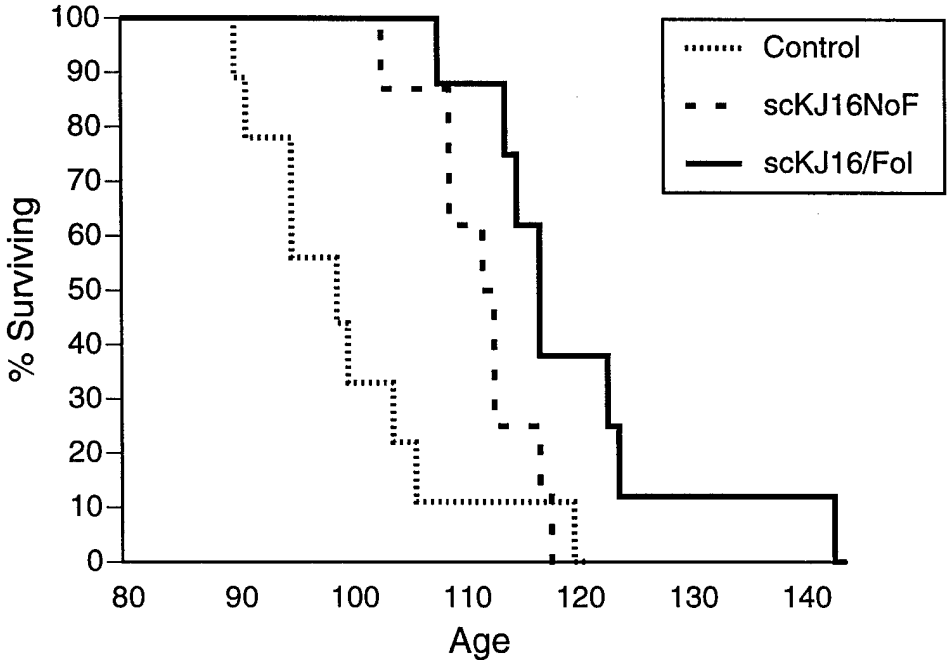


FIGURE 4 – Prolongation of survival of SV11 mice following antibody treatments. Experimental mice were treated with SEB (100 μg i.p.) at day 84 followed over the next 13 days by 4 treatments with either scFv-KJ16 (25 μg i.p.) or folate/scFv-KJ16 (25 μg i.p.). Mice treated with SEB/scFv-KJ16 lived longer than controls ($p < 0.05$) and mice treated with SEB and the folate conjugate of scFv-KJ16 lived longer than mice treated with the non-conjugate form of the antibody ($p < 0.05$).

evaluate if sustained or more extensive T-cell infiltration can lead to higher survival rates.

SV11 tumors arise because SV40 large T antigen interferes with p53 and pRB, and this etiology may be similar to some human brain tumors. SV40 has been isolated from pediatric choroid plexus tumors and ependymomas (Bergsagel *et al.,* 1992). SV40-induced tumors are immunogenic if transplanted into congeneic C57BL/6 mice (Mylin *et al.,* 1995), but SV11 mice are apparently immunologically tolerized to the tumors during development, as are other SV40 transgenic strains. We have transplanted the choroid plexus tumors to an S.C. location in SV11 mice, and the tumors were not rejected (data not shown). At this point, we have not determined whether either of the treatments that prolonged survival generated a tumor-specific T-cell response. It may be that the folate conjugation and targeting of T cells involves both redirected killing of tumor cells and the generation of tumor-specific T cells that have overcome tolerance, enhancing a process that may have occurred in animals treated with SEB and scFv-KJ16 without folate.

We have investigated the relationship of the blood-brain barrier and blood cerebrospinal fluid barrier to this treatment strategy. As the tumors develop, there are multiple foci in the lateral ventricles and 4th ventricle. The smaller tumors have an intact barrier (as revealed by the absence of murine IgG and exclusion of Evans blue/albumin), whereas the larger tumors have lost the barrier. Although there is heterogeneity in the density of the T-cell infiltrate in different foci, it is only partly accounted for by the blood-brain barrier. For example, there are definite small tumors with an apparently intact blood-brain barrier that also have infiltrating T cells. With adoptively transferred T cells incubated with an IgG form of anti-CD3 antibody/folate conjugate and infused directly into the brain, we have observed retention of T cells and a similar prolongation of survival (data not shown).

With systemic treatment, we observed few T cells associated with regions of normal choroid plexus, which also expresses the high-affinity folate receptor on its apical surface (Patrick *et al.,* 1997; Weitman *et al.,* 1992) and no infiltration of T cells into other normal tissues that have lower levels of the high-affinity folate receptor such as kidney and lung. Consistent with this finding, there have been no signs of obvious neurological problems in tumor-bearing or normal C57BL/6 mice treated with the bispecific agents described in this study, nor, apparently, in ovarian cancer clinical trials using conventional anti-FR bispecific antibodies (Mezzanzanica *et al.,* 1991). It may be that the blood cerebrospinal fluid barrier is intact in the choroid plexus, and the tumor vasculature is partially compromised even in small tumors. In this regard, there is a substantial body of literature supporting the notion that activated T cells can penetrate the blood-brain barrier, but penetration is enhanced through a partially disrupted barrier (Fabry *et al.,* 1995). This ability to penetrate the blood-brain barrier suggests that strategies to enhance T-cell activity in brain tumors may have advantages over strategies that rely on the penetration of antibodies alone.

The general approach of coupling a small high-affinity ligand to antibodies against immune effector cells (Chen *et al.,* 1995; Kranz *et al.,* 1995) should be widely applicable to any type of cancer in which a high-affinity ligand against a tumor-associated molecule can be identified. The folate/antibody approach may work more effectively against tumors such as ovarian carcinomas that do not have the complication of the blood-brain barrier and that may be more accessible by T cells. It is also likely that the ligand-targeting approach described here can be used to enhance other aspects of a T-cell response. In particular, folate conjugation to antibodies against T-cell co-stimulatory receptors such as CD28 may elicit enhanced T-cell activation at the site of the tumor, thereby combining activation and targeting while limiting systemic toxicity (Jung *et al.,* 1991).

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# INTRACEREBRAL BISPECIFIC LIGAND-ANTIBODY CONJUGATE INCREASES SURVIVAL OF ANIMALS BEARING ENDOGENOUSLY ARISING BRAIN TUMORS

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**Bispecific antibodies capable of simultaneously binding a tumor surface antigen and the T-cell receptor/CD3 complex are capable of inducing polyclonal immune effector cells to destroy targeted tumor cells. Bispecific antibody immunotherapies have shown some promise against tumors of hematopoietic origin such as lymphomas, but use of bispecific antibodies for the treatment of solid tumors has been less fully explored. To test the preclinical potential of bispecific antibody therapy against an endogenously arising solid brain tumor, we have utilized a novel variation of conventional bispecific antibodies, referred to as bispecific ligand-antibody conjugates, to target choroid plexus tumors. The bispecific ligand-antibody conjugate described in this study is a chemical conjugate between an anti-CD3 monoclonal antibody (MAb) and folic acid, the ligand for a high-affinity surface receptor expressed on the surface of choroid plexus tumors. SV11 mice transgenic for SV40 large T antigen and its promoter develop solid choroid plexus tumors in the brain. We demonstrate that choroid plexus tumor cells are susceptible *in vitro* to cytolysis mediated by cytotoxic T cells in the presence of the bispecific ligand-antibody conjugate in a folate-inhibitable manner. Adoptive immunotherapy studies demonstrate the potential benefits of the bispecific ligand-antibody conjugate *in vivo*. The bispecific conjugate is capable of retaining adoptively transferred T lymphocytes specifically within tumor tissue for periods of up to at least 1 week. Further, following intracerebro-ventricular injection of bispecific conjugate and splenocytes containing activated cytotoxic T cells, T cells were observed to penetrate to interior regions of the tumor. A single treatment of adoptively delivered activated effectors and bispecific conjugate into the brain ventricles was insufficient to produce significant increases in survival of SV11 mice, but repeated treatment through indwelling cannulas prolonged survival of animals treated with activated effectors and bispecific ligand-antibody conjugate compared to animals treated with activated effectors or saline alone. Our results demonstrate that the SV11 model may be useful for preclinical evaluation and optimization of bispecific ligand-antibody conjugate treatments of solid tumors. *Int. J. Cancer* 78:470–479, 1998.**
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Tumors may evade immune recognition via a number of mechanisms. The lack of appropriate antigen presentation and/or costimulation by tumors is one means of preventing specific T-lymphocyte recognition. In addition, the microenvironment in which a tumor arises may actively suppress the initiation or execution of a competent specific immune response to tumor.

The brain has classically been considered an immune privileged site. The blood-brain barrier (BBB) is thought to prevent trafficking of unactivated lymphocytes through brain parenchyma, and the decreased surveillance may lower the probability of specific recognition of brain tumors. Molecular factors blocking the activation of immune effectors cells [*e.g.*, transforming growth factor-β (TGF-β) or prostaglandin $E_2$] are released by cells in the brain including microglia, astrocytes and certain types of brain tumors (Fabry *et al.*, 1995). Systemic release of immunosuppressive factors by some brain tumors may further hamper the ability of the immune system to mount or maintain a response to tumor and, in some cases, may cause systemic immunosuppression (Van Meir, 1995). In light of these concerns, therapeutic strategies that overcome endogenous lymphocyte activation barriers may be useful.

One means of circumventing possible endogenous activation barriers is to activate effector lymphocytes *ex vivo*. Some clinical studies have focused on adoptive transfer of lymphokine activated killer (LAK) cells (Hayes *et al.*, 1995; Smith *et al.*, 1996). Some patients showed improvement when treated with LAK cells, but the recurrence of primary malignancy was high. This finding may be due to the polyclonal nature of the LAK cells that largely are not specific to the tumor under treatment. Adoptive transfer studies may benefit from approaches that redirect polyclonal effector cells to specifically lyse tumor cells.

A strategy that has been shown to redirect cytolytic action of polyclonal cytotoxic T cells (CTL) specifically against various tumors is bispecific antibodies. Simultaneously specific for a tumor surface antigen and an immune effector triggering molecule, bispecific antibodies can juxtapose CTL to tumor cells and mediate the specific redirection of polyclonal immune effectors against tumor cells. Bispecific antibodies circumvent the requirement for tumor antigen presentation to CTLs by directly signaling via immune effector surface molecules (*e.g.*, T-cell receptor/CD3 complex) responsible for stimulating cytotoxic action. Variations of the bispecific antibody strategy have been shown to be successful in numerous animal models and are currently being employed in clinical trials (*e.g.*, Canevari *et al.*, 1995).

Whereas most animal studies employing bispecific antibodies have concentrated on hematopoietic tumors, solid tumors may be a more difficult challenge. Targeting and maintaining sufficient immunotherapeutic reagents or active immune effector cells in the interior of tumors may be an obstacle to bispecific antibody treatment of solid tumors. As with some other immunotherapeutic strategies (*e.g.*, transfection of tumor cells with immune activating cytokines), reported success against solid tumors using bispecific antibody therapy is generally limited to preventing the growth of small tumor burdens transplanted into animal models rather than rejecting an established, solid tumor of endogenous origin.

One model for endogenously arising brain tumors is SV11 mice that are transgenic for large T antigen (Tag) and its associated promoter derived from the simian virus 40 (SV40) genome (Van Dyke *et al.*, 1985, 1987). SV11 mice develop choroid plexus tumors (CPT) with 100% penetrance and become moribund at approximately 100 days of age (Van Dyke *et al.*, 1987). The mechanism of tumorigenesis in SV11 mice involves direct inhibition of tumor suppressors p53 and pRB by Tag in choroid plexus epithelial cells. The reason for the selective expression of Tag in the choroid plexus epithelium is unknown. SV40 Tag sequences have been found in a majority of pediatric ependymomas and half of the

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pediatric CPTs tested (Bergsagel *et al.*, 1992). In fact, intact virions of SV40 have been isolated from some of these tumors (Lednicky *et al.*, 1995). Speculation of a viral etiology for pediatric ependymomas and CPTs suggests that CPTs which develop in SV11 mice may be analogous to the development of their human counterparts and hence useful for the study of various immunotherapeutic regimes aimed at eliminating solid tumors.

Previously, we described a novel bispecific antibody targeting strategy where the small ligand folic acid was chemically coupled to anti-T-cell receptor (TCR) antibodies (Kranz *et al.*, 1995). These bispecific ligand-antibody conjugates demonstrated specific and sensitive ability to redirect CTLs to lyse tumor cells expressing the high-affinity folate receptor (FR) *in vitro*. FR has been suggested as a potential tumor-associated antigen useful for immunotherapeutic targeting of certain human neoplasms (Bolhuis *et al.*, 1992). Among neoplasms expressing FR are CPT, ependymomas, >95% of ovarian carcinomas and approximately 30% of mammary adenocarcinomas (Buist *et al.*, 1993; Coney *et al.*, 1991; Ross *et al.*, 1994). SV11 CPTs express FR and thus may be useful for testing and optimizing the bispecific ligand-antibody conjugate strategy *in vivo* (Patrick *et al.*, 1997). We have shown that treatment of SV11 mice with a single-chain Fv antibody against Vβ8 TCRs conjugated with folate caused T-cell infiltration of the tumors and significantly prolonged survival (Roy *et al.*, 1998). In the present study, we used an anti-CD3 antibody conjugated to folic acid that targets all T cells, and delivered the bispecific ligand-antibody conjugate directly into the brain.

We first determined whether an anti-CD3 antibody/folate conjugate is capable of redirecting CTL against CPT cells. The anti-murine CD3 antibody 2C11 conjugated to folate was capable of redirecting both monoclonal and polyclonal CTIs to specifically lyse CPT cells *in vitro*. Adoptive transfer of polyclonal CTL and 2C11/folate conjugate directly in the lateral ventricles of the brain of SV11 animals led to retention of T lymphocytes in CPT within the interior of the tumor. In contrast, rapid clearance of CTL from the brain was observed in the absence of bispecific ligand-antibody conjugate. Finally, continued adoptive therapy of SV11 mice with CTL and bispecific ligand-antibody conjugate conferred a significant improvement in survival compared to animals treated with CTL or saline alone.

## MATERIAL AND METHODS

### *Mice*

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in animal facilities at the University of Illinois. The SV11 transgenic line was obtained from T.A. Van Dyke (University of North Carolina). SV11 males heterozygous for Tag were mated to C57BL/6 females. Progeny were screened by polymerase chain reaction (PCR) for the presence of Tag (Patrick *et al.*, 1997). Prior to use in adoptive transfer treatment regimes, mice were placed on low-folate chow for a period of at least 1 week to reduce the level of serum folates that could potentially compete with the bispecific ligand-antibody conjugate for binding to FRs on CPT. All studies described were approved by the Laboratory Animal Care Advisory Committee and conducted in accordance with NIH guidelines for the care and handling of laboratory animals in experimental studies.

### *Cell lines and antibodies*

All cell lines were incubated in a humidified incubator at 37°C and 5% $CO_2$. F2-MTX$^r$A, a non-adherent DBA/2-derived erythroleukemia line (Brigle *et al.*, 1991), was maintained in RPMI 1640 medium containing 5 mM HEPES, 10% (vol/vol) heat-inactivated fetal bovine serum, 1.3 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 units/ml streptomycin. Cytotoxic T-lymphocyte clone 2C, a mouse alloreactive cell line specific for $L^d$, was maintained in the same RPMI medium described above and supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5% α-methylmannoside and mitomycin C-treated BALB/c spleen cells ($L^d$) as stimulators (Kranz *et al.*, 1984). Hybridoma 2C11 (Leo *et al.*, 1987), a hamster IgG specific for the murine CD3ε subunit, was purified from ascites by ammonium sulfate precipitation and protein A-Sepharose. Hybridoma 37.51 (Gross *et al.*, 1992), a hamster IgG-secreting line specific for murine CD28, was cultured in serum-free RPMI 1640 medium and purified by passage over a protein G-Sepharose column. Rabbit antiserum to murine FR (kindly provided by Dr. K. Brigle) and normal rabbit antiserum (kindly provided by S. Miklasz) were utilized in flow cytometry without further processing.

### *Flow cytometry*

CPT cells were mechanically dissociated through wire mesh and separated by sedimentation into individual cells and small aggregates. Red blood cells were lysed by incubation in lysing buffer (0.14 M $NH_4Cl$, 0.017 M Tris, pH 7.2) for 5 min at 37°C. Isolated CPT cells were incubated with polyclonal rabbit antiserum to murine FR followed by fluorescent goat anti-rabbit secondary (Kirkegaard and Perry, Gaithersburg, MD) or secondary antibody alone. Cells were analyzed on a Modified Coulter EPICS 753 flow cytometer (Coulter, Miami, FL) with Cyclops version 3.14 software for the percentage of CPT cells expressing FR. Splenocytes were labeled with a combination of fluorescent anti-CD4, CD8 and CD69 primary antibodies (Pharmingen, San Diego, CA). Cells were analyzed on a Coulter XL-MCL flow cytometer with Coulter System II software. Dead cells were excluded on the basis of high- or low-angle light scatter.

### *Preparation of antibody/folate conjugate*

Conjugation of folic acid to purified 2C11 was performed as described previously (Kranz *et al.*, 1995). Briefly, folic acid (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 6.7 mM. EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; Pierce, Rockford, IL] was added to a final concentration of 33.5 mM (1:5 folate/EDC ratio) and incubated for 30 min at room temperature in the dark. Reacted folate/EDC was added to 2C11 antibody (1 mg/ml) in 0.1 M MOPS, pH 7.5, at a 100:1 folate/antibody ratio. Further incubation for 1 hr at room temperature in the dark was followed by passage over a Sephadex G-25 (Sigma) column equilibrated in phosphate-buffered saline (PBS). The excluded-peak fractions containing conjugated antibody were pooled and analyzed spectrophotometrically for determination of folate density. Densities averaged 4–8 folates/antibody. 2C11/folate bispectific ligand-antibody conjugates were stored at 4°C in the dark.

### *Cytotoxicity assays*

Mechanically dissociated CPT cells or F2-MTX$^r$ A cells were labeled with 50 μl of $^{51}Cr$ (2.5 mCi/ml) for 1 hr at 37°C, washed repeatedly in folate-free RPMI 1640 containing 5% (vol/vol) fetal bovine serum and subsequently used in 96-well plate cytotoxicity assays at $2 \times 10^4$ cells/well. 2C11/folate conjugate was added to triplicate wells at 150 ng/ml. For folate-inhibition studies, free folate was added at various final concentrations ($2.5 \times 10^{-7}$ to $2.5 \times 10^{-11}$ M). Activation of CTL clone 2C effector cells was as described above and added at an effector to target ratio of 10:1. Activation of splenocytes from C57BL/6 or SV11 mice was induced by an intravenous (i.v.) injection of 10 μg 2C11 antibody. The spleen was removed from these mice 48 hr later (optimal time for CTL activation, data not shown). Splenocytes were isolated by disruption through wire mesh and red blood cells were lysed by incubation in lysing buffer for 5 min at 37°C. Remaining splenocytes were washed 3 times in folate-free RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal bovine serum and added at effector to target ratios of 100:1, 31:1, 10:1, 3:1 or 1:1 as indicated. Plates were incubated at 37°C for 4 hr in a humidified 5% $CO_2$ incubator. Culture supernatants were removed for γ counting. Specific $^{51}Cr$-release was calculated by standard methods [% specific $^{51}Cr$-release = (experimental counts − spontaneous counts)/[maximal counts − spontaneous counts) × 100].

In vivo *activation of splenocytes for adoptive transfer*

C57BL/6 mice were injected i.v. with 10 μg anti-CD3 antibody 2C11 and harvested as described under cytotoxicity assays. Splenocytes were washed with PBS rather than folate-free RPMI 1640. In cases were splenocytes and bispecific ligand-antibody conjugate were simultaneously transferred, splenocytes and the conjugate were incubated on ice for at least 30 min.

*T-lymphocyte retention in CPT* in vivo

SV11 mice aged 90–100 days and having been placed on low-folate chow for a period of not less than 1 week were anesthetized with 1.7 mg/20 g body weight ketamine and 250 μg/20 g body weight xylazine in sterile PBS injected intraperitoneally (i.p.). Mice scalps were shaved and prescrubbed with betadine. Surgery was performed using aseptic techniques. An incision of approximately 1 cm was made along the mid-sagittal plane of the scalp. A stereotaxic device was employed to determine coordinates 1 mm caudal and 1 mm lateral of bregma. The skull was perforated with a drill. Intracerebroventricular (ICV) injections (either saline alone, $2 \times 10^6$ activated splenocytes, or $2 \times 10^6$ activated splenocytes and 100 ng 2C11/folate conjugate) were delivered 2.5 mm deep of dura (location of superior horn of lateral ventricle) in a total volume of 5–10 μl at a rate of 2 μl/min, using a Hamilton syringe with an attached 28 gauge needle. Following a 5 min latency postdelivery, the syringe was removed and the skull sealed with bone wax. The scalp was sutured or sealed with Vetbond (3M, Minneapolis, MN). Animals were anesthetized with ketamine/xylazine at 24 hr following ICV delivery. Animals were perfused with acetic acid, zinc, formalin (AZF) fixative (Newcomer Supply, Middleton, WI) at room temperature. Brain, spleen and thymus were removed from each animal and bathed in fixative overnight at room temperature.

*Tissue preparation and histochemistry*

AZF-fixed brains were blocked into 5–6 transverse sections at designated points (mid-IVth ventricle, rostral cerebellum and approximately every 2.5 mm further rostral) the day following fixation and paraffin embedded along with spleen and thymus samples from the same mouse. All brain sections, spleen and thymus to be analyzed by immunohistochemistry were cut and mounted as 3 μm sections on the same slide. Slides were deparaffinized in xylene, rehydrated to PBS, blocked with Superblock (Pierce) and incubated overnight with rabbit anti-human CD3 (murine CD3 cross-reactivity; DAKO, Carpinteria, CA) at 4°C. Primary antibody was followed by biotinylated goat anti-rabbit and subsequently by avidin-biotin-horseradish peroxidase (HRP) (Vector, Burlingame, CA). Nickel-cobalt-enhanced diaminobenzidine (DAB; Pierce) chromagen was used for detection. Slides were counterstained in methyl green, dehydrated and coverslipped with Permount. Sections of spleen and thymus served as both positive and negative controls with respect to regions labeling positive for the presence of CD3. Analysis of tumor area was determined using NIH Image at ×25 magnification. Individual T cells associated with tumor were counted at ×100 magnification for density calculations (T cells/unit area).

*Apoptosis tissue stains*

Tissue was prepared as above. Adjacent tissue sections were placed on separate slides for comparison of T lymphocyte presence and apoptosis. A TUNEL assay kit (Trevigen, Gaithersburg, MD) was utilized to detect apoptosis. Briefly, tissue was deparaffinized in xylene and an ethanol series followed by several washes in distilled water. Proteinase K (20 μg/ml) was added to each slide, plastic coverslipped and incubated at room temperature for 15 min. Slides were immersed in 2% $H_2O_2$ in methanol for 5 min before transferring slides to labeling buffer. Manufacturer's labeling reaction mixture was added to each slide, plastic coverslipped and incubated in a 37°C humid chamber for 1 hr. Following labeling reaction, slides were placed in stop buffer for 5 min, washed 3 times with PBS over a 5 min period and incubated with streptavidin-HRP in PBS for 10 min. Slides were washed again in PBS before addition of metal-enhanced DAB chromagen. Following a 5 min incubation, slides were rinsed repeatedly in distilled water and subsequently counterstained with methyl green. Brain parenchyma, thymus and spleen from experimental animals served as apoptosis controls as well as control slides provided by the manufacturer.

*Time course of T-lymphocyte retention in CPT* in vivo

The protocol for adoptive transfer of splenocytes and 2C11/folate conjugate was repeated exactly as for T lymphocyte retention at 24 hr, but animals were killed at various time points following ICV delivery ranging from 24 hr to 7 days. Four animals receiving activated polyclonal splenocytes and 2C11/folate conjugate were killed at 24 hr, one animal receiving activated polyclonal splenocytes and 2C11/folate conjugate was killed at each successive time point, 3 animals receiving activated polyclonal effectors alone were killed at 24 hr and 3 untreated animals were killed at time zero, as a control for background density of T cells normally found within CPT. Cross-sectional areas of lateral ventricle CPT from at least 4 separate regions of cortex were calculated on NIH Image, and the number of T cells within those areas quantified to yield T-cell density within tumor.

*Treatment of SV11 mice with a single adoptive transfer of activated splenocytes*

SV11 mice aged 85–90 days, on low-folate chow for at least 1 week, were injected ICV as described above. Mice were distributed to treatment groups randomly with the proportions of each group matched for sex where possible. Mice received either an average of $3.5 \times 10^6$ activated splenocytes and 100 ng 2C11/folate ($n = 11$), $3.5 \times 10^6$ activated splenocytes ($n = 7$) or PBS alone ($n = 11$). In instances where 2C11/folate was added to effectors for ICV delivery, incubation was at least 30 min on ice prior to ICV delivery. Mice were monitored daily until 1 of 3 criteria establishing morbidity was met, at which time the mouse was killed, perfused, and brain, spleen and thymus were prepared for immunohistochemistry as described above.

*Clinical evaluation of mice*

Animals were weighed daily and monitored for level of activity and vestibular integrity. Loss of greater than 25% of pretreatment body weight, lack of responsiveness to external stimuli or carrying head at an improper angle or inability to right self were established as sufficient criterion for morbidity classification.

*Statistical methods*

T-cell densities were compared using the Student's *t*-test. Survival analyses were based on age at first sign of morbidity according to the above criteria. Ages were entered into a standard Kaplan-Meier survival plot. Statistical *p* values were calculated using the log-rank method on JMP software (SAS Institute, Cary, NC). Mean and median ages at morbidity are reported.

*Comparison of* in vivo- *and* in vitro-*activated splenocytes*

Unactivated splenocytes were harvested from C57BL/6 mice and depleted of red blood cells with lysing buffer as described above. Splenocytes were washed in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum. Splenocytes were placed in 24-well tissue culture plates at $1.5 \times 10^6$ cells/well with 100 ng/ml anti-CD3 antibody 2C11 and 5 μg/ml to 8 μg/ml anti-CD28 antibody 37.51 or as indicated. Splenocytes were incubated for 72 h at 37°C in a humidified 5% $CO_2$ incubator. All cell preparations were >95% viable. Splenocytes activated *in vivo* as described above were compared to splenocytes activated *in vitro* by flow cytometry for percent composition of $CD8^+$ cells. Cytotoxic assays similar to those described above using the $FR^+$ cell line $F^2$-$MTX^r$A as tumor target were used to compare killing efficiencies of the 2 T-lymphocyte activation methods. The $ER_{50}$ (effector to target ratio giving half-maximal lysis) was calculated by linear regression of data points in the linear region of the curve

and was used to compare the lytic potential of *in vivo*- and *in vitro*-activated splenocytes.

*Treatment of SV11 mice with multiple adoptive transfers of* in vitro*-activated splenocytes*

Mice aged 65–70 days were prepared for surgery as described above. Permanent cannulas were placed bilaterally in the skull of SV11 mice. Knotted PE-50 tubing was inserted bilaterally 1.6 mm caudal and 0.6 mm lateral of bregma to a depth of 3 mm below the skull. Cannulas were held in place with dental cement anchored to a "00" machine screw placed approximately 5 mm rostral of the left cannula. In some cases, stylets fashioned from 28 gauge stainless steel tubing were used to prevent cannula occlusion. Animals were allowed a 2 week period to recover from surgery. Morbidity due to surgery was minimal (<5%). At approximately 85 days of age, mice were injected bilaterally (when possible) with $5 \times 10^5$ to $2.5 \times 10^6$ splenocytes and 100 ng 2C11/folate conjugate (n = 18), $5 \times 10^5$ to $2.5 \times 10^6$ splenocytes (n = 11) or saline (n = 6) in a total volume of 10 µl. Splenocytes were activated *in vitro* (as described above) with 100 ng/ml 2C11 and 0.5–1 µg 37.51. Splenocytes were harvested and washed 3 times in PBS prior to ICV injection.

## RESULTS

*FR expression by CPT cells*

The first objective was to determine whether CTL could be redirected by the 2C11/folate conjugate to lyse cells from a solid CPT. To establish the proportion of CPT cells that are positive for FR expression and may serve as potential targets for the bispecific ligand-antibody conjugate targeting strategy, a preparation of dissociated CPT cells from an SV11 mouse was labeled with a polyclonal serum to murine high-affinity FR (Fig. 1). Flow cytometric analysis indicated that a large fraction (>97%) of the isolated tumor cells expresses detectable FR.

*Susceptibility of CPT cells to monoclonal CTLs* in vitro

To test the susceptibility of these CPT cells to CTL-mediated lysis, dissociated CPT cells were incubated with the CTL clone 2C and 2C11/folate conjugate (150 ng/ml) in a standard $^{51}$Cr-release assay. Various concentrations of free folate were added to establish if redirection of cytotoxic activity is specifically mediated through FR expressed by the CPT cells. As shown in Figure 2, CTL clone 2C is capable of lysing CPT cells in a folate-dependent manner. CPT cell incubation with CTL clone 2C in the absence of 2C11/folate conjugate resulted in no detectable lysis (data not shown), further suggesting that the 2C11/folate conjugate is responsible for the redirection of 2C toward FR-expressing cells.

*Susceptibility of CPT cells to polyclonal CTLs* in vitro

Having established that CPT cells were susceptible to CTL clone 2C, we next determined whether activated splenocytes containing an activated population of polyclonal CTLs derived from C57BL/6 or SV11 mice could be redirected to lyse CPT cells *in vitro*. C57BL/6 or SV11 mice were injected i.v. with 10 µg of 2C11 antibody, which produces an active splenocyte subpopulation of CTL capable of cytolysis 24–48 hr later as measured by standard $^{51}$Cr-release assays (data not shown). Splenocytes were harvested from these animals 48 hr after 2C11 administration and utilized in a standard $^{51}$Cr-release assay against CPT cells. Figure 3*a* demonstrates that in the absence of 2C11/folate conjugate, both activated and unactivated populations of splenocytes have negligible lytic activity toward CPT cells in a 4 hr assay. Figure 3*b* shows that the presence of 2C11/folate conjugate and activated CTL from either C57BL/6 or SV11 mice can be redirected to lyse CPT cells, while CTLs not preactivated are incapable of inducing lysis in a 4 hr assay. These data indicate that CPT cells are susceptible to lysis by activated polyclonal CTLs from either SV11 or C57BL/6 mice and that 2C11/folate conjugate is a capable mediator of redirecting the lytic potential of these CTLs. Similar to the data presented for monoclonal CTL 2C, the redirection of lytic activity by these *in vivo*-activated CTLs was inhibitable by addition of free folate (data not shown).

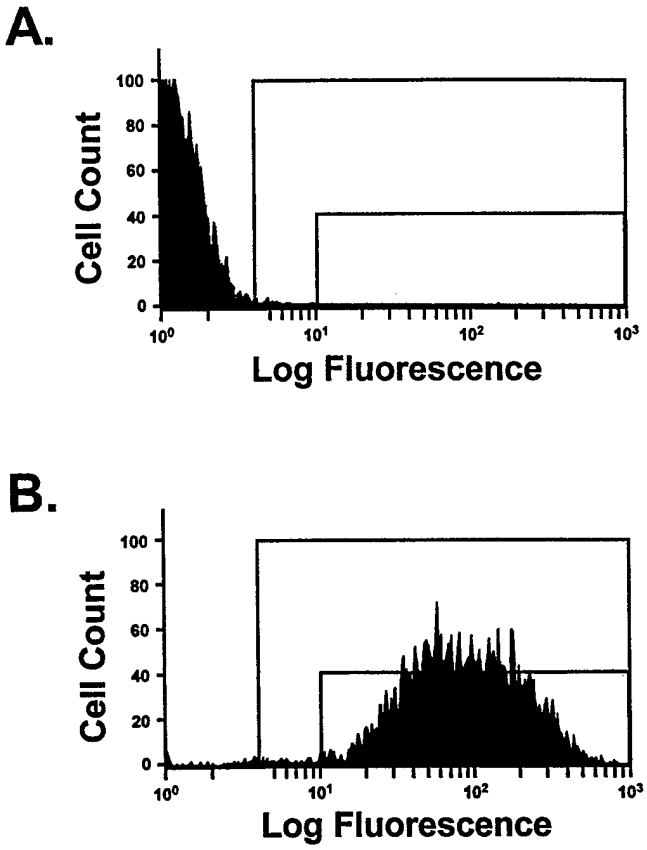



FIGURE 1 – Flow cytometric analysis of high-affinity FR expression. Mechanically dissociated SV11 CPT cells were labeled in the presence of normal rabbit antiserum *(a)* or a rabbit polyclonal serum to murine FR *(b)*. Each was followed by an immunofluorescent goat anti-rabbit secondary antibody.

*2C11/folate conjugate retains T cells within CPTs* in vivo

To assess the action of the bispecific ligand-antibody conjugate at juxtaposing activated polyclonal CTLs against CPT *in vivo*, we utilized preparations of activated C57BL/6 splenocytes described above incubated with 2C11/folate conjugate or vehicle. An average of $2 \times 10^6$ cells suspended in PBS was adoptively transferred directly into the superior lateral ventricles in a volume of 5–10 µl. Animals were killed 24 hr following ICV delivery. Splenocytes injected in the absence of 2C11/folate conjugate showed few T cells associating with CPT after 24 hr (Fig. 4*b*). In contrast, when splenocytes were injected with 2C11/folate conjugate, a significant increase in the density of T cells was present throughout CPT 24 hr later ($p < 0.01$ *vs.* splenocytes without 2C11/folate conjugate) (Fig. 4*a*). Many of the T cells associating with CPT appear to have penetrated to interior depths of the solid tumor. Further, in both 2C11/folate conjugate-treated and untreated animals, few T cells are found in normal brain parenchyma at 24 hr and those observed were typically clustered along myelin tracts or adjacent to vessels, suggesting an outward emigration.

Adjacent tissue sections were analyzed separately for the presence of $CD3^+$ cells or apoptosis 24 hr after ICV administration of activated splenocytes and 2C11/folate conjugate (Fig. 4*c,d*). Apoptotic cells were clustered variously throughout tumor tissue. Juxtaposed to many of the apoptotic cells are $CD3^+$ cells, but the apoptotic cells do not simultaneously stain for the CD3 antigen. Apoptosis was not detected among normal choroid plexus epithe-

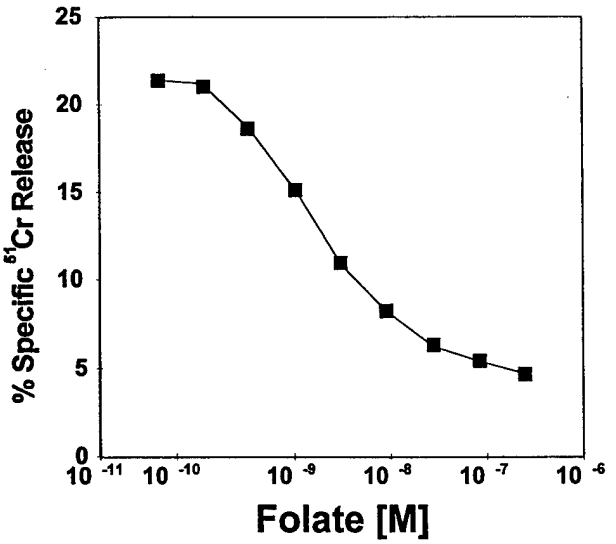



FIGURE 2 – Specific redirection of CTL clone 2C cytotoxic activity against CPT cells by 2C11/folate conjugate. To test the susceptibility of CPT cells to lysis by CTLs mediated through redirection by the 2C11/folate conjugate, $^{51}$Cr-labeled CPT cells were incubated over a 4 hr period with CTL 2C, 150 ng/ml 2C11/folate conjugate and various concentrations of competing free folate. Results presented are the mean of triplicate samples.

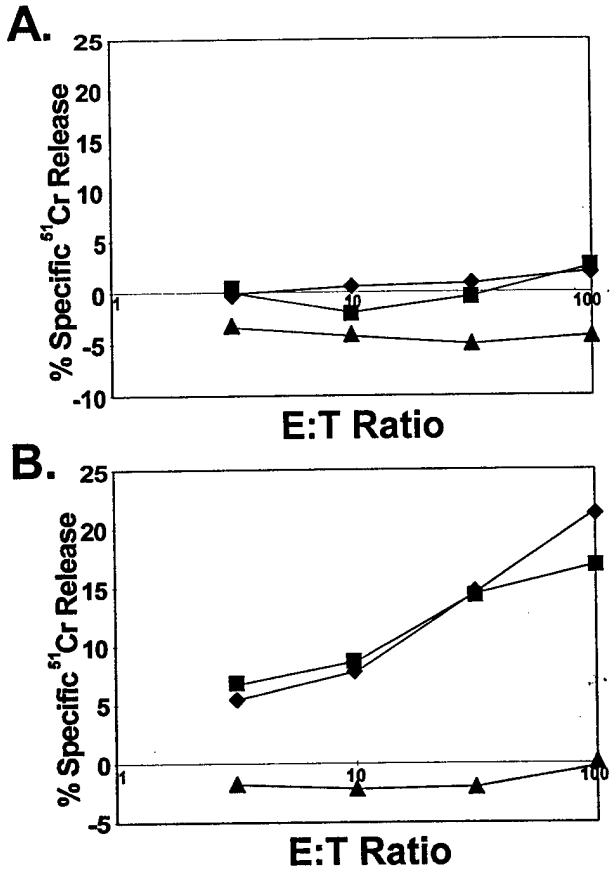



FIGURE 3 – Specific redirection of activated splenocytes to lyse $^{51}$Cr-labeled CPT cells. The capacity for *in vivo*-activated effector CTLs among activated splenocytes to be redirected to lyse CPT cells by 2C11/folate conjugate was tested. Activated C57BL/6 (■), SV11 (♦) or unactivated SV11 (▲) splenocytes were incubated at various effector to target ratios for 4 hr in the absence *(a)* or presence *(b)* of 2C11/folate conjugate and CPT cells. Specific $^{51}$Cr-release mediated by 2C11/folate conjugate redirection of CTL activity was inhibited by free folate (data not shown).

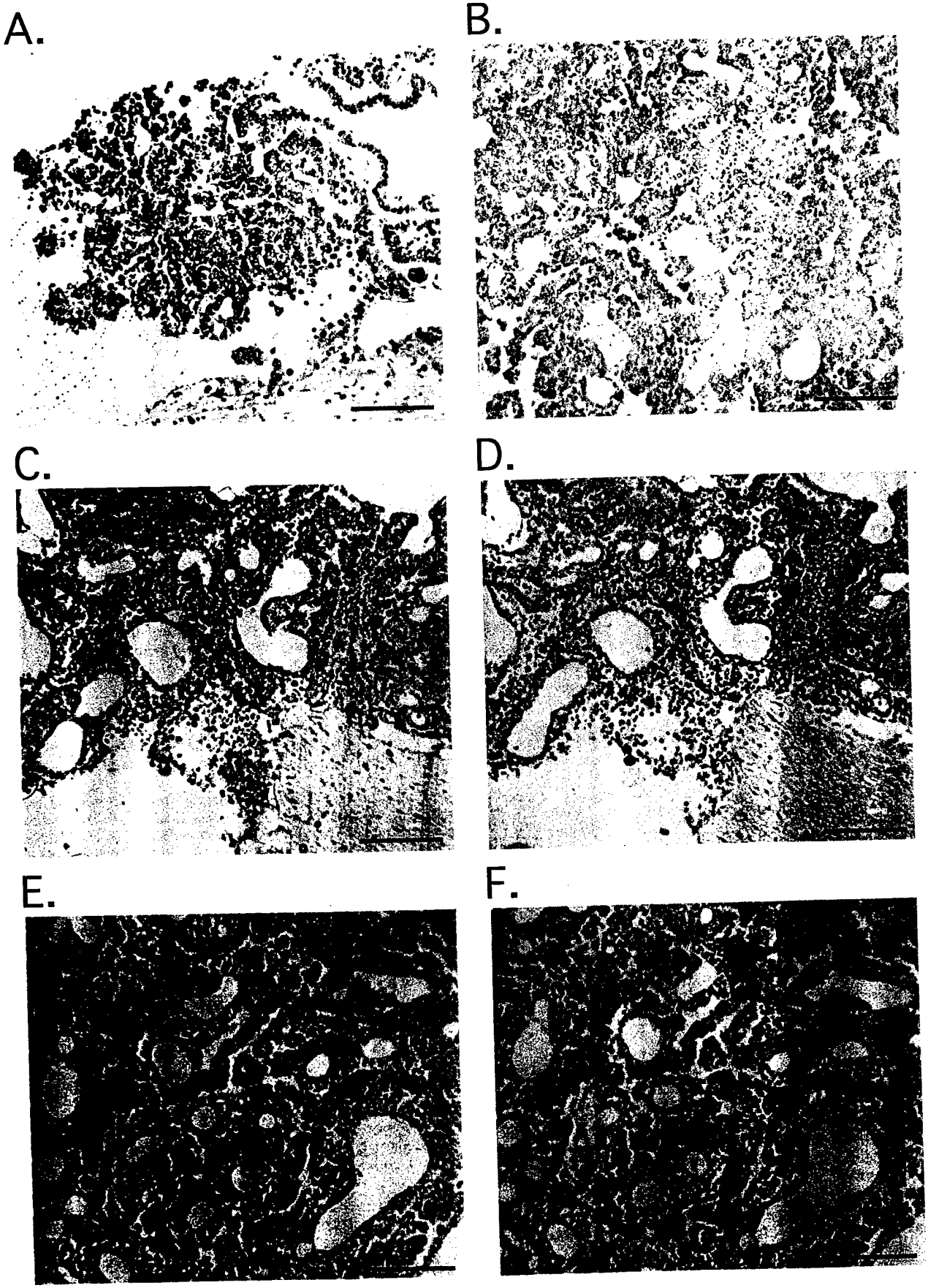
lium, brain parenchyma or the ependymal lining of the brain ventricles (data not shown). Marginal apoptosis of CPT cells was observed in animals treated with activated splenocytes without 2C11/folate conjugate or saline alone (Fig. 4*e,f*). Further, apoptosis was not observed in CPT of animals treated with activated splenocytes and 2C11/folate conjugate 48 hr or longer after ICV transfer (data not shown).

*2C11/folate conjugate retains T lymphocytes in CPT for periods of up to 1 week*

A time course study revealed the duration that the 2C11/folate conjugate retained T cells in tumor tissue. SV11 mice were injected ICV with *in vivo*-activated splenocytes in the presence or absence of 2C11/folate conjugate. Subsequently, animals were perfused at various time points and brain sections stained for the presence of CD3. Figure 5 illustrates the mean density of T cells in 2C11/folate conjugate-treated animals over time compared with animals receiving T cells without 2C11/folate conjugate or with untreated SV11 animals. Corroborating results shown in Figure 4*b*, at 24 hr post-ICV delivery the density of T cells in CPT tissue of animals receiving no 2C11/folate conjugate at 24 hr was low, with an average of less than 25 T cells/mm$^2$. Similarly, the baseline presence of T cells within untreated SV11 animals was low, averaging 3 T cells/mm$^2$. In marked contrast, at 24 hr post-ICV delivery, animals receiving T cells with 2C11/folate conjugate had elevated levels of T cells among tumor tissue averaging over 600 T cells/mm$^2$ ($p < 0.01$ presence *vs.* absence of 2C11/folate conjugate) and in certain regions T-cell densities were over 1,500 T cells/mm$^2$. Further, elevated numbers of T cells can be found in tumor tissue for up to 7 days compared to the no 2C11/folate conjugate 24 hr time point. The density of T cells in CPT 24 hr following delivery of activated splenocytes and unlabeled 2C11 antibody was similar to the density of delivering activated splenocytes alone (data not shown).

To control for the possibility that the T cells observed in the sections were not those adoptively transferred but immigrating endogenous cells, we analyzed the effects of injecting 2C11/folate conjugate or unlabeled 2C11 MAb alone into the lateral ventricles of SV11 animals. Injection of 2C11/folate conjugate ICV without splenocytes results in the immigration of a negligible density of T cells into CPT (data not shown). Even under circumstances where a preactivating dose of anti-CD3 antibody was administered systemically to SV11 animals 24 hr prior to ICV delivery of

FIGURE 4 – Retention of T cells in CPT and apoptosis. Animals were infused ICV with activated splenocytes in the presence *(a)* or absence *(b)* of 2C11/folate conjugate and killed 24 hr later. Tissue sections from multiple brain regions were stained for CD3. T cells are present throughout CPT tissue when 2C11/folate conjugate was administered *(a)*, but T cells have migrated out by 24 hr if 2C11/folate conjugate was not administered *(b)*. Relatively few T cells were observed in normal brain parenchyma. Adjacent sections from an animal treated with 2C11/folate and activated splenocytes killed 24 hr after adoptive transfer ICV were stained for CD3 *(c)* and apoptosis *(d)*. Again, 2C11/folate conjugate retained T cells specifically within CPT *(c)*. Cells staining for CD3 *(c)* are juxtaposed to apoptotic cells *(d)*. Apoptosis was not observed in normal brain parenchyma or choroid plexus. Animals not receiving 2C11/folate conjugate revealed few retained T cells in CPT at 24 hr *(e)* or apoptosis of tumor tissue on adjacent sections *(f)*. Scale bar for *a–f*: 100 μm.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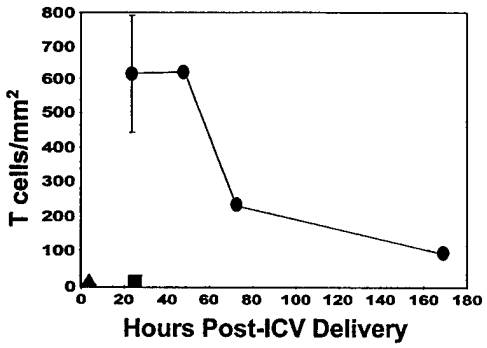


FIGURE 5 – T-cell retention in CPT following ICV delivery. Approximately $2 \times 10^6$ activated splenocytes were injected ICV in the presence (●) or absence (■) of 2C11/folate conjugate and compared with T-cell density in CPT of untreated SV11 animals (▲). SV11 animals were killed at various time points following injection, perfusion fixed and brains mounted for anti-CD3 immunohistochemistry. Total tumor area of lateral ventricle CPT of either treated or sham control animals was calculated from 3–5 brain sections of differing regions and the T cells quantified were associated with tumor. Vertical bars indicate SD. The number of animals used at each time point: 0 hr, n = 3; 24 hr, n = 4 for each; 48 hr, n = 1; 72 hr, n = 1; 168 hr, n = 1. Activated splenocytes with 2C11/folate differed from both untreated controls and activated splenocytes without conjugate, $p < 0.01$.

2C11/folate conjugate in attempts to promote CTL activation and surveillance, a negligible increase in density of T cells was observed infiltrating into CPT. Collectively, the observations indicate that the most probable origin of the T cells within sections of the CPT is that which is adoptively transferred.

*Survival analysis of SV11 animals with a single adoptive transfer of activated splenocytes and 2C11/folate conjugate*

SV11 mice received a single ICV injection of *in vivo*-activated splenocytes and 2C11/folate conjugate, splenocytes alone or PBS. Survival results indicated that administration of splenocytes and 2C11/folate conjugate in a single bolus ICV did not confer a statistically significant therapeutic advantage over administering T cells alone ($p > 0.05$) or sham controls ($p > 0.05$). While animal longevity was not extended, fewer animals appear to become moribund at younger ages (85–100 days) when treated with T cells (with or without 2C11/folate conjugate) compared to sham controls.

*Comparison of* in vivo- vs. in vitro-*activated splenocyte effectors*

Flow cytometric analysis of *in vivo*-activated splenocytes harvested for ICV delivery demonstrates that the effector population of activated $CD8^+$ cells accounted for only 4–9% of total splenocytes compared with an average of 15–20% in a resting spleen (Fig. 6*a,b*). CD69, an early marker indicative of T-lymphocyte activation (Allison *et al.*, 1995), was present on approximately 70–80% of $CD4^+$ or $CD8^+$ cells in animals receiving 2C11, but on less than 2% of T lymphocytes in animals not receiving 2C11. Thus, injection of $3.5 \times 10^6$ splenocytes resulted in fewer than $3.5 \times 10^5$ $CD8^+$ cytotoxic T cells actually delivered and fewer still likely capable of cytolysis. Due to the low proportion of $CD8^+$ cells rendered with *in vivo* activation, splenocytes were activated *in vitro* with a combination of anti-CD3 (2C11) and anti-CD28 (37.51) antibodies to expand the proportion of CTL effectors in the overall cell population. Splenocytes were incubated with 100 ng/ml 2C11 antibody and various doses of 37.51 to determine the optimal level for producing active CTL effectors. Activation of splenic T cells with CD28 costimulation after 72 hr consistently expanded the $CD8^+$ population to 60–70% of total cells regardless of the 37.51 dose tested (approximately 10 times the percentage of $CD8^+$ cells found among *in vivo*-activated splenocytes). Representative flow cytometric data for the *in vitro*-activated splenocytes for $CD8^+$ cells are shown in Figure 6*c*. CD69 expression is not observed on the predominance of T lymphocytes following *in vitro* activation most likely as a result of the relatively transient expression of this marker.

We tested the relative effectiveness of *in vitro*- and *in vivo*-activated splenocytes at lysing a FR-expressing target mediated by 2C11/folate redirection. Similar to *in vivo*-activated CTLs, *in vitro*-activated CTLs demonstrate the capacity to lyse the FR-expressing cell line F2-MTX$^r$A in the presence of 2C11/folate conjugate (Fig. 7*a,b*). Comparison of the lytic potential of these 2 effector populations revealed that the $ER_{50}$ (effector to target ratio giving half-maximal lysis) is approximately 6- to 10-fold lower for *in vitro*-activated effectors (10 times fewer *in vitro*-activated splenocytes compared to *in vivo*-activated splenocytes). Given that the percentage of $CD8^+$ cells among *in vitro*-activated splenocytes is approximately 10-fold greater than that among the *in vivo*-activated splenocytes, the resulting 10-fold difference in the $ER_{50}$ likely is a function of CTL number and not differences in CTL cytotoxic potency.

*Survival analysis of SV11 animals treated with multiple adoptive transfers of* in vitro-*activated polyclonal effectors and 2C11/folate conjugate through indwelling cannulas*

Two groups of mice receiving *in vitro*-activated cells every 3 or 5 days, either with 2C11/folate conjugate (n = 18) or without (n = 11), were compared to a group of animals receiving saline (n = 6). Survival analysis demonstrated that cotreatment of CPT with activated splenocyte effector cells and 2C11/folate conjugate improved survival over administration of T cells alone ($p = 0.001$) or saline ($p = 0.003$) (Fig. 8). Mean time to morbidity of animals treated with T cells alone was 98 days (SD 1, median 98) while animals receiving 2C11/folate conjugate survived an average of 107 days (SD 2, median 107) indicating that the presence of 2C11/folate conjugate conferred a therapeutic advantage. Mean time to morbidity of saline controls was 99 days (SD 1, median 98).

## DISCUSSION

The aim of our study was to determine whether a bispecific ligand-antibody conjugate could produce a significant improvement in survival of animals with an endogenously arising solid brain tumor, when delivered directly to the tumor. The first objective was to determine whether CPT cells would be susceptible to bispecific ligand-antibody conjugate-mediated cytolysis by polyclonal effector cells derived from SV11 mice or congeneic C57BL/6 mice. Some types of brain tumors secrete substances that inhibit the activation of T cells, *e.g.*, TGF-β. The possibility exists that the CPT in the SV11 animal provides a locally immunosuppressive microenvironment. Unactivated polyclonal splenocytes from SV11 animals showed no detectable lytic activity against CPT, suggesting that either the SV11 animal is tolerant to the presence of tumor or that the tumor may be locally or systemically immunosuppressing the mice. However, anti-CD3-activated polyclonal effectors from SV11 or C57BL/6 mice could be induced to lyse CPT in the presence of 2C11/folate conjugate, demonstrating that if there is systemic immunosuppression, it can be overcome. Further, these same activated polyclonal effectors from SV11 mice did not lyse CPT in the absence of 2C11/folate conjugate, suggesting that SV11 mice do not harbor expanded antitumor clones among splenocytes. Folate competition of CTL-mediated lysis demonstrated that the 2C11/folate conjugate was responsible for the cytotoxic interaction. In addition, incubating target cells with active CTL and unlabeled 2C11 antibody did not lead to cytolysis of target cell lines *in vitro* (data not shown).

When administered *in vivo*, the 2C11/folate conjugate was capable of retaining T lymphocytes within CPT tissue, whereas these same effectors quickly cleared from the brain in the absence of 2C11/folate conjugate. These data suggest that the 2C11/folate

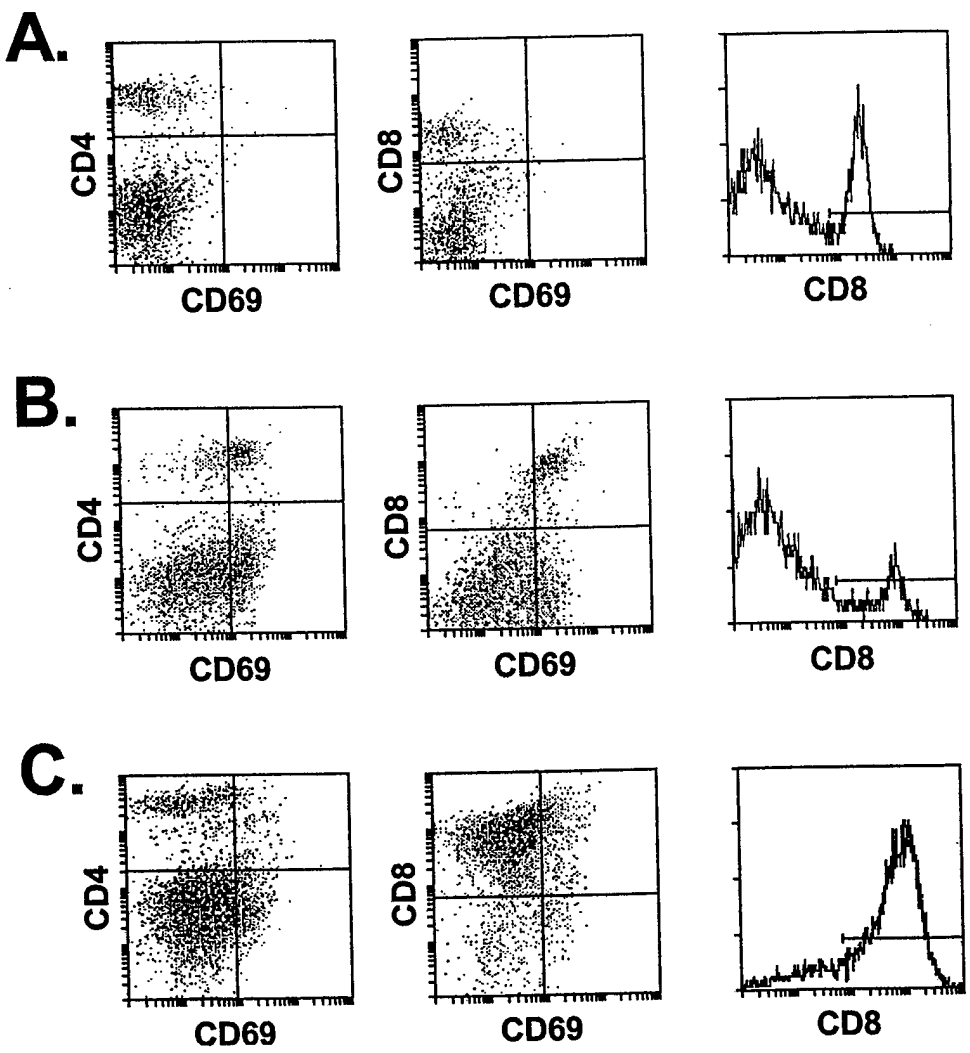


FIGURE 6 – Flow cytometric analysis of activated splenocytes. Resting *(a)* or *in vivo*-activated *(b)* splenocytes were labeled with a combination of labeled anti-CD4, CD8 and CD69 antibodies. Resting splenocytes have $CD4^+$ and $CD8^+$ populations of approximately 25–35% and 15–20%, respectively, and low expression of the early activation marker CD69. Twenty-four hours following a 10 μg injection of 2C11, the majority of $CD4^+$ and $CD8^+$ cells are positive for CD69, indicating an activated state, although the percentage of $CD8^+$ cells has markedly decreased among splenocytes (<8%). Splenocytes activated *in vitro (c)* were similarly labeled with a combination of anti-CD4, CD8 and CD69 antibodies following a 72 hr incubation period with 100 ng/ml 2C11 and 0.5 mg/ml 37.51. The percentage of $CD8^+$ cells has expanded (68%). The early activation marker CD69 largely is not present on these cells, indicating marker turnover rather than an unactivated state.

conjugate is capable of contributing to the prevention of the normal migratory tendency of activated T lymphocytes. The data also indicate that the 2C11/folate conjugate is capable of retaining T lymphocytes in CPT tissue for periods of up to 1 week at higher than background densities.

The first treatment protocol involved adoptive delivery of a single injection of activated splenocytes and 2C11/folate conjugate. The animals were treated at 85–90 days of age, a point at which the CPTs typically have progressed to grade III and IV neoplasms. The survival of SV11 animals treated in this fashion did not significantly differ from that of animals treated with activated splenocytes alone or saline-injected animals. Considering the relative density of T cells adhering to CPTs in the time course analysis, calculations indicate that perhaps 1 T cell is present for every 4–10 CPT cells at 24–48 hr post-ICV delivery. The low proportion of $CD8^+$ cells among *in vivo*-activated splenocytes and the relatively short duration of peak retention of T-cell density suggested the need for enriching the proportion of $CD8^+$ CTLs among the splenocyte pool as well as devising a means to deliver multiple injections of CTL and 2C11/folate conjugate.

Activating splenocytes *in vitro* greatly expanded the CTL effector population. Although CTLs activated in this fashion are not more potent against CPT cells than the *in vivo*-activated counterparts, more CTLs could be delivered per injection. Delivering multiple infusions of splenocytes and 2C11/folate conjugate through indwelling cannulas increased SV11 animal survival. Activating splenocytes *in vitro* results in an effector CTL population that is precoated with unlabeled 2C11 antibody (flow cytometric results, data not shown). Repeated delivery of splenocytes alone into the lateral brain ventricles in these experiments actually includes delivery of 2C11 antibody. Because splenocytes precoated with 2C11 did not confer a therapeutic advantage over animals treated with saline, the 2C11/folate conjugate was responsible for conferring the beneficial therapeutic effect.

To further characterize CTL action against CPT *in vivo* and possible side effects to choroid plexus epithelium or healthy brain

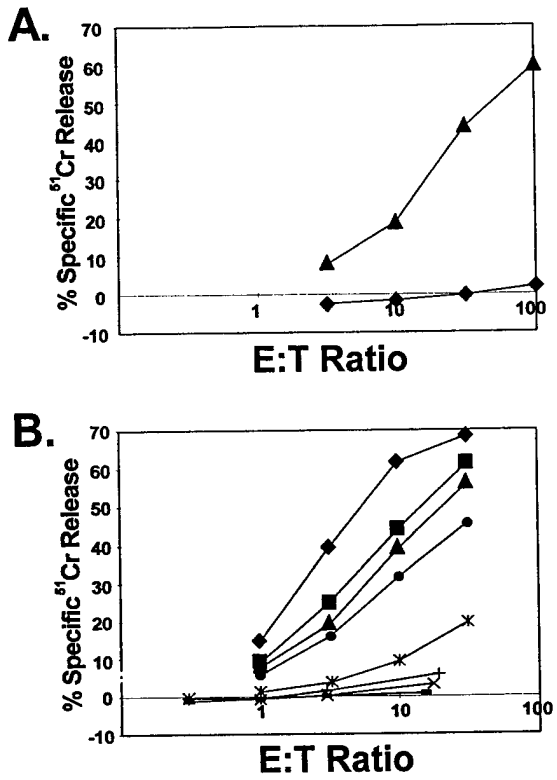


FIGURE 7 – Comparison of *in vivo*- and *in vitro*-activated splenocyte retargeting. The $FR^+$ tumor target line $F^2$-$MTX^rA$ and various effector to target (E:T) ratios of each effector population were incubated with 150 ng/ml 2C11/folate. *In vivo*-activated effectors *(a)* were tested either in the presence (▲) or absence (♦) of 2C11/folate conjugate at E:T ratios of 100:1, 30:1, 10:1 and 3:1. Splenocytes activated *in vitro (b)* were stimulated with 100 ng/ml 2C11 and various concentrations of 37.51:5 μg/ml (♦), 1 μg/ml (■), 0.2 μg/ml (▲), 0.04 μg/ml (●) and 0.008 μg/ml (*). Splenocytes were also incubated with 100 ng/ml 2C11 alone (+), 5 μg/ml 37.51 alone (×) or media alone (—). E:T ratios tested for all *in vitro*-activated splenocytes were 30:1, 10:1, 3:1 and 1:1. Half-maximal lysis ($ER_{50}$) was achieved at an E:T of approximately 30:1 by *in vivo*-activated splenocytes, but an $ER_{50}$ of less than 5:1 for *in vitro*-activated splenocytes.

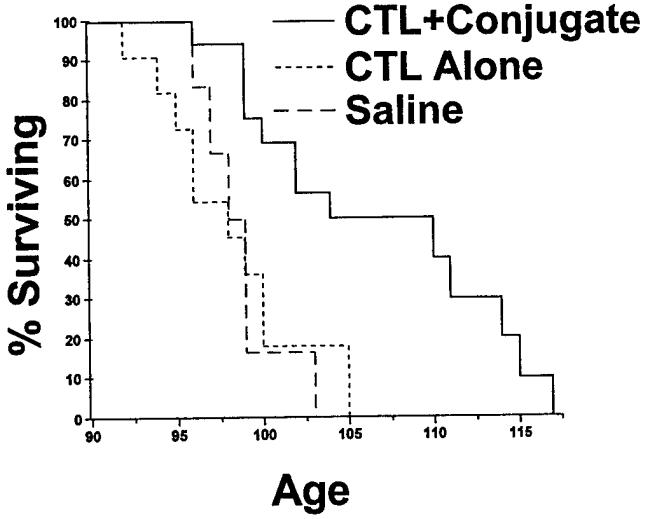


FIGURE 8 – Survival of cannulated SV11 mice treated ICV with multiple injections of *in vivo*-activated splenocytes and 2C11/folate conjugate. SV11 animals were injected ICV at approximately 85 days of age and every 3–5 days thereafter until morbidity. Animals received saline (——) (n = 6), preactivated splenocytes (---) (n = 11) or preactivated splenocytes and 1 μg 2C11/folate conjugate (—) (n = 18). Animals were assessed daily for alteration in body weight and vestibular complications. A 25% decrease in pretreatment body weight or inability to right self served as criterion for morbidity. Animals treated with 2C11/folate conjugate and activated splenocytes lived significantly longer than animals treated with saline ($p = 0.003$) or activated splenocytes without conjugate ($p = 0.001$).

parenchyma, we performed apoptotic assays of tissue sections adjacent to those stained for CD3. Untreated SV11 animals commonly have evidence of a slight amount of background apoptosis in CPT tissue. Apoptosis of CPT cells of SV11 animals treated with saline or activated splenocytes alone was not appreciably different from untreated SV11 animals. We observed a greater extent of apoptotic staining in CPT of SV11 animals receiving 2C11/folate conjugate and activated splenocytes. Adjacent slide sections stained for apoptosis or the CD3 antigen showed apoptotic cells to be juxtaposed to cells staining for CD3, suggesting that the 2C11/folate conjugate may be redirecting CTL action against CPT cells. Apoptosis of non-tumor tissue was not observed in any of the treatment group animals nor in normal C57 mice treated under similar conditions.

T cells delivered into the lateral ventricles with 2C11/folate conjugate were found to penetrate tumors located in all regions of the lateral or third ventricles, but IVth ventricle tumors were typically devoid of T-cell presence, suggesting that T cells are not penetrating the cerebral aqueduct following ICV delivery. A number of SV11 animals receiving CTL and 2C11/folate conjugate had small or negligible tumor loads in the lateral ventricles at later survival times, compared with the typically large lateral ventricle tumors of animals not receiving 2C11/folate conjugate, and large IVth ventricle tumors.

A potential cause for concern regarding the ICV treatment regime is that normal choroid plexus epithelium expresses the high-affinity folate receptor as do CPTs. Normal choroid plexus has a polarized distribution of FR concentrated on the apical surface facing the ventricular lumen directly exposed to the injected T cells and 2C11/folate conjugate (Patrick *et al.*, 1997). In numerous cases, T cells were found associated with normal choroid plexus, although predominately in the presence of 2C11/folate conjugate. While the choroid plexus was sometimes altered in morphology compared with that of untreated animals, we were unable to detect damage to normal choroid plexus in treated mice, either by gross morphology or presence of apoptotic cells. We do not know why epithelial cells appeared resistant to T-cell-mediated lysis. Similarly, C57 mice treated with activated T cells and 2C11/folate conjugate did not show any signs of short-term (12–24 hr) or long-term (up to 1 year) toxicity to normal choroid plexus.

Given the transgenic nature of the CPT, we did not expect any of the treatment regimes to produce a complete cure. All cells of the choroid plexus epithelium may serve as potential origins of tumorigenesis. SV11 animals commonly have multiple CPT foci in the lateral and IVth ventricles at morbidity. A more realistic goal in this model is comparing increases in mean time to morbidity rather than expecting long periods of disease-free remission.

While treatment of SV11 animals with multiple injections of splenocytes and 2C11/folate conjugate improved animal survival, there is clearly room for improvement. An important issue to address is the length of time retained CTLs remain active and capable of cytolysis within the CPT. While T cells were observed adhering to CPT for periods beyond 1 week post-ICV delivery, the activational status of these cells is unknown. Considering that primary T-cell activation is a transient process for an individual T cell, CTLs are unlikely to remain active for extended periods of

time (Speiser *et al.*, 1997). In addition, activation of CTL with 2C11 leads to a down-regulation of CD3 that may have reduced the potential efficacy of the bispecific ligand-antibody conjugate strategy for treatment of CPTs. As a potential means of promoting greater longevity of CTL activation, the application of additional bispecific ligand-antibody conjugates targeting costimulatory molecules (*e.g.*, folate/anti-CD28, folate/anti-LFA-1) may simultaneously enhance primary activation or permit reactivation of adoptively transferred cells. An additional strategy may be to deliver blocking anti-CTLA-4 monovalent antibody fragments to reduce possible CTL down-regulation (Allison *et al.*, 1995).

Targeting sufficient numbers of activated effectors to tumor has been a primary concern for most immunotherapeutic strategies. The high affinity of the FR for folate and the apparent capability of the 2C11/folate conjugate to keep T cells retained in tumor for periods of up to 1 week suggest that attaching a high-affinity ligand to antieffector cell antibodies may be an effective means of targeting and retaining effector cells in tumor regions. The incorporation of bispecific ligand-antibody conjugates selectively stimulating costimulatory molecules on effector cells may promote sustained reactions to tumor, and perhaps the recruitment of endogenous effectors.

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# BISPECIFIC AGENTS TARGET ENDOGENOUS MURINE T CELLS AGAINST HUMAN TUMOR XENOGRAFTS

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**A variety of immunological approaches to cancer treatment are currently being explored. These include strategies designed to enhance or redirect the activity of T cells against tumors. Bispecific antibodies comprise a class of agents capable of redirecting T cells by binding to a tumor antigen and the T-cell receptor (TCR). *In vivo* pre-clinical testing of bispecific antibodies against human tumors has to date been limited to the use of immunodeficient mice that receive the bispecific agent, activated human effector T cells, and human tumor cells. In this report, we show that TCR transgenic/RAG-1 knockout mice (TCR/RAG) serve as a unique model allowing endogenous T cells to be redirected against transplanted human tumors. The findings show that TCR/RAG mice (i) accepted transplants of human tumors, including the folate-receptor-positive tumor line KB; (ii) contained endogenous cytotoxic T lymphocytes that could be activated *in vivo* with an antigenic peptide recognized by the transgenic TCR; (iii) rejected human tumors after treatment with the activating peptide and bispecific agents that contained folic acid co-valently linked to an anti-TCR antibody. Successful rejection was achieved with folate conjugates of Fab or scFv fragments. Treatment with activating agents and bispecific conjugates resulted in the complete eradication of freshly transplanted tumors as well as significantly prolonging the survival of mice bearing established solid tumors. Our results highlight the importance of including T-cell-activating modalities in combination with bispecific antibodies. Additionally, we introduce a system that allows endogenous T cells to be redirected against human tumor xenografts and in which the T cells may be followed *in vivo* by use of a clonotypic marker.**
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Over the past decade, bispecific antibodies have been used in attempts to redirect the immune system against a variety of different types of cancer. Bispecific antibodies that are designed to redirect the activity of T cells typically recognize both a tumor antigen and the T-cell-receptor (TCR/CD3) complex (Fanger *et al.,* 1993; Renner and Pfreundschuh, 1995). *In vitro* and *in vivo* studies with many different tumor cell types have demonstrated the potential of this approach. However, results have also shown that optimal activity of these agents will most likely require combination treatment with T-cell-activating agents such as anti-CD3 and anti-CD28 antibodies or superantigens such as Staphylococcal enterotoxin B (Bluestone *et al.,* 1992; Mazzoni *et al.,* 1996). These findings are not surprising in the light of current understanding of the signals required to induce resting T cells to fully activated T cells. That is, the co-stimulatory molecule CD28, and perhaps LFA-1 under some circumstances are required for the complete activation of naive T cells. Earlier studies with bispecific antibodies in fact suggested the importance of LFA-1 in the lysis of tumor cells (Braakman *et al.,* 1990; Goedegebuure *et al.,* 1990). Co-stimulatory signals (signal two) are not normally provided by bispecific antibodies, since in general these exhibit specificity for the TCR or CD3 molecules and thus provide signal one alone.

Even in combination with T-cell-activating agents, bispecific-antibody therapy of solid tumors has been particularly difficult (Bakacs *et al.,* 1995). Part of the problem has been the inability to induce sufficient T-cell infiltration into a solid tumor or to sustain the activity of T cells at tumor sites. Adequate pre-clinical study of anti-human bispecific antibodies in these respects has been impossible, since these agents must be tested in immunodeficient animal models, due to the barriers of xenotransplantation. Accordingly, the bispecific antibodies are typically administered together with activated human T cells and the human tumor into either nude or SCID mice. In these animal models, it is impossible to evaluate the complexities that are associated with the *in vivo* activation of T cells, the migration of endogenously activated T cells, or the ability to induce and sustain such T cells at tumor sites.

The development of transgenic and knockout mice has provided an opportunity to design novel animal models that could be used in these studies. For example, genetically defined immunodeficient mice were produced by knocking out the recombination-activating-gene 1 (Mombaerts *et al.,* 1992). These mice are unable to rearrange immunoglobulin or T-cell-receptor genes, and therefore mature lymphocytes of the B- and T-cell lineages do not develop. In contrast to these immunodeficient mice, TCR transgenic mice express the rearranged α- and β-chain genes from a specific T-cell clone and therefore contain a predominantly monoclonal population of T cells. For example, mice that are transgenic for the TCR from the mouse alloreactive ($L^d$) CTL clone 2C bear the same TCR on 60 to 90% of their $CD8^+$ T cells (Sha *et al.,* 1988). However, the remaining T cells express endogenous TCR, thus these mice are capable of mounting an immune response against various antigens. Consequently, 2C TCR transgenic mice will not accept allogeneic or xenogeneic tumor transplants.

Since the 2C CTL does not itself recognize human tumors, we reasoned that 2C TCR transgenic mice crossed with *rag*-1 knockout mice could provide a useful system to evaluate the parameters involved in optimal targeting of endogenous T cells against transplanted human tumors. This report characterizes the 2C TCR/RAG-$1^{-/-}$ system as a model for the treatment of human tumors with bispecific agents that recruit endogenous T cells against the tumor, and characterizes the *in vivo* response of the transgenic T cells to activation and to redirecting agents.

This system also has the advantage that CTL clone 2C has been studied by many investigators to identify the ligands that the 2C TCR recognizes and to explore the requirements for activation. Several peptides recognized by the 2C TCR when bound to the negatively selecting MHC class I ($L^d$) or the positively selecting MHC class I ($K^b$) have been described. Initially, the octomer peptide p2C was identified as the natural peptide recognized by 2C when bound to $L^d$ (Udaka *et al.,* 1992). A synthetic peptide SIYRYYGL when bound to $K^b$, has been shown to be recognized by CTL 2C (Udaka *et al.,* 1996). There is now also considerable information about the ability of peptides and anti-CD3 and anti-CD28 antibodies to stimulate resting T cells isolated from 2C TCR transgenic mice.

The human tumor model chosen to explore the TCR/RAG$^{-/-}$ system is the folate-receptor-positive line KB. The folate receptor (FR) is expressed on over 90% of ovarian tumors, and conventional bispecific antibodies against this tumor antigen were among the

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first to be tested clinically (Luiten *et al.,* 1997; Ross *et al.,* 1994). In some clinical studies of ovarian-cancer patients receiving i.p. administration of bispecific antibodies, complete remissions have been observed *in situ,* but no systemic effects were observed (Bolhuis *et al.,* 1992; Canevari *et al.,* 1995). To explore strategies that might improve the systemic effects, we and others have begun to evaluate a variety of novel therapeutics directed against the high-affinity FR (Leamon *et al.,* 1993; Mathias *et al.,* 1996). These potential therapeutics contain folate attached to cytotoxic moieties or to imaging agents. Folate attached to anti-TCR antibodies can redirect the activity of T cells against $FR^+$ tumors (Kranz *et al.,* 1995). We have shown that the smallest bispecific antibodies yet described could also be engineered by attaching folate to the Fab fragment and scFv forms of an anti-TCR antibody (KJ16) (Cho *et al.,* 1997). As an extension of these studies, we show here that these folate conjugates are also potent agents in the CTL-mediated lysis of human tumor xenografts. Furthermore, the folate conjugates are shown to be effective *in vivo* as surrogate T-cell antigens on transplanted tumor cells. The findings show that the TCR/$RAG^{-/-}$ model can now be used to guide the discovery of improved T-cell-specific agents. As such, this system should facilitate more rapid and effective pre-clinical development of various T-cell-modulating drugs.

## MATERIAL AND METHODS

### *Antibodies*

Fab fragments and scFv from the anti-TCR antibody KJ16 (rat IgG), which is specific for the Vβ8 region of the TCR, were generated and purified as described (Cho *et al.,* 1995). Briefly, scFv was re-folded from inclusion bodies and purified by Q-Sepharose ion exchange chromatography. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure (Cho *et al.,* 1997; Kranz *et al.,* 1995). Each conjugate preparation was analyzed for biological activity in a cytotoxicity assay prior to use in mice. 1B2, a mouse IgG1 monoclonal antibody (MAb) that is clonotypic for the 2C TCR, was used in cytotoxicity assays and for flow analysis. Anti-Thy-1.2 conjugated to phycoerythrin was purchased from PharMingen (San Diego, CA).

### *SIYRYYGL peptide*

The SIYRYYGL peptide (Udaka *et al.,* 1996) was synthesized on an Applied Biosystems 430A instrument (Foster City, CA) using standard F-moc chemistry at the University of Illinois Biotechnology Center (Urbana, IL). The peptide was purified by reverse-phase HPLC with a C18 column and analyzed for purity by mass spectrometry and for concentration by quantitative amino-acid analysis.

### *Mice*

Experiments were performed with 2C TCR/RAG-$1^{-/-}$ mice (Manning *et al.,* 1997) between 6 and 12 weeks of age. Mice were bred and housed in barrier cages at the University of Illinois animal-care facility.

### *Cell lines*

KB was derived from a human epidermoid carcinoma and expresses high levels of folate receptor (Mathias *et al.,* 1996). These cells were maintained in folate-free DMEM containing 10% fetal bovine serum. P815, a DBA2-derived H-$2^d$ mastocytoma tumor line against which the alloreactive T cell clone 2C was initially selected, was maintained in complete RPMI 1640 containing 10% fetal bovine serum (Kranz *et al.,* 1984). RMAS, an H-$2^b$ tumor line that does not express the high-affinity folate receptor, was maintained in complete RPMI 1640 containing 10% fetal bovine serum. CTL clone 2C was maintained in complete RPMI 1640 containing 10% fetal bovine serum, 10% culture supernatant from rat splenocytes stimulated with concanavillin A, and mitomycin-C-treated BALB/c splenocytes as stimulator cells.

### *Cytotoxicity assays*

Cytoxicity was examined in standard $^{51}Cr$-release assays. Briefly, target cells were labeled with $^{51}Cr$ for 1 hr at 37°C. Target cells ($10^4$ per well) of a 96-well plate were incubated with various numbers of effector cells for 4 hr. Supernatants were harvested and assayed for specific lysis according to the formula: % specific lysis = (cpm experimental − cpm spontaneous)/(cpm maximal − cpm spontaneous). Spontaneous lysis was less than 20% of maximal lysis. Analysis of redirected lysis used a slightly modified protocol. Folate-free DMEM medium was used with KB cells as the target. Cultured 2C or *in vitro*-activated T cells were used as effector cells. The folate/antibody conjugates were added to triplicate wells at various concentrations diluted in folate-free medium. In some cases, free folate was added to a final concentration of 1.2 μM.

### *Flow cytometry*

Flow cytometry was performed at the University of Illinois Flow Cytometry Facility using a Coulter (Hialeah, FL) Epics XL instrument. Peritoneal lavage was performed and the exudate cells were washed and counted. Cells ($2 \times 10^5$) were incubated with the appropriately labeled antibody in 50-μl volume on ice. Cells were washed and re-suspended in 400 μl before analysis.

### In vivo *treatments*

*SIYRYYGL peptide activation.* Mice were injected i.p. with SIYRYYGL peptide in 200 μl of PBS. For experiments with the KB tumor, the peptide was given 24 hr before each antibody administration. All peptide and antibody agents were sterile-filtered prior to *in vivo* use.

*Tumor transplantation.* For s.c. tumor transplants, each mouse was anesthetized and hair was removed from the injection site before KB tumor cells ($10^7$, $3 \times 10^6$ or $2.6 \times 10^6$ in 200 μl PBS) were injected. These injections were made on the left side of the back near the scapula. Vernier calipers were used to take 2 perpendicular measurements of the tumor twice weekly, and measurements were averaged to determine mean tumor diameter. When the tumors reached a mean diameter of 20 mm, animals were killed with $CO_2$. For i.p. tumor transplants, $5 \times 10^6$ cells in 1 ml of PBS were injected. These mice were monitored daily and killed when the tumors enlarged the abdomen and the mice became moribund.

*Bispecific conjugate therapy.* Except where indicated, mice were treated with 2.5 nmol SIYRYYGL peptide i.p. on day −1 and tumor cells on day 0. Tumor cells were suspended in PBS alone or in PBS containing the KJ16 antibody preparation. Antibody-treatment groups included: folate/Fab (10 or 20 μg), folate/scFv (1, 10 or 20 μg). Control antibody groups included scFv without attached folate (20 μg) and Fab fragments without attached folate (10 μg). For treatments initiated on day 0, KB cells were mixed with the antibody prior to injection. A second activating peptide treatment was administered on day 3, followed on day 4 with the appropriate antibody (i.p. 200 μl for s.c. transplants and 1 ml for i.p. transplants). Additionally, an established i.p. treatment group was injected with tumor cells 2 days prior to antibody therapy. These established tumor groups received antibody treatments on days 2 and 6, preceded by peptide activation on days 1 and 5.

*Analysis of T-cell infiltration.* Mice were injected i.p. with $2 \times 10^6$ tumor cells on day −2, received peptide (2.5 nmol) on day −1, and on day 0 received KJ16 Fab or KJ16 folate/Fab (1 μg). Peritoneal exudate cells were collected on day +3, counted, analyzed by flow cytometry and tested for cytolytic activity.

### *Statistics*

Differences in survival between treatment groups was determined using the Kaplan-Meier survival method within the SAS JMP software.

## RESULTS

### *Growth of human tumors in TCR/RAG mice*

RAG-1 knockout mice have been shown to lack B and T cells, and do not exhibit the leakiness of SCID mice (Mombaerts *et al.,* 1992). We have observed that RAG-1$^{-/-}$ mice are capable of accepting various human tumor xenografts (data not shown). In order to develop an animal model that would allow endogenous T cells to be re-targeted against such transplanted human tumors, a 2C TCR transgenic mouse was crossed with a RAG-1$^{-/-}$ mouse. TCR/RAG$^{-/-}$ progeny from back-crossed mice exhibit an immune system in which essentially 100% of the peripheral T cells bear the 2C TCR and 95% are CD8$^{+}$, with the remainder CD4$^{-}$CD8$^{-}$ (Manning *et al.,* 1997). CTL 2C was originally produced as an alloreactive (L$^{d}$) specific clone. Because 2C is incapable of recognizing any human tumor line that has been tested (Kranz *et al.,* 1984; and data not shown), we reasoned that 2C TCR/RAG transgenic mice might also accept some human tumor xenografts, as do RAG-1$^{-/-}$ mice. Of several different human tumor lines tested (SKBR-3, SKOV-3, BT474, KB), all except SKBR-3 grew consistently as s.c. transplants (data not shown).

The folate-receptor-positive line KB was selected for further study, in order to evaluate whether various bispecific folate/antibody conjugates (Cho *et al.,* 1997; Kranz *et al.,* 1995) would be capable of redirecting the activity of the endogenous 2C CTL against the human tumor. Immunohistology of KB tumors that grew after i.p. or s.c. injection confirmed that these tumors continue to express the folate receptor after transplantation into TCR/RAG mice (data not shown). Folate receptors also appeared to be distributed uniformly at the surface of KB cells. KB tumor xenografts are locally aggressive, but appear to be only weakly metastatic. The tumors rapidly outgrow their blood supply, resulting in relatively well-demarcated margins with a necrotic core. S.c. tumor grafts rarely grew outside of the s.c. space. I.p. growth usually involved several independent foci adherent to the peritoneal wall or organs. These tumors did not appear to be metastatic beyond the abdomen but occasionally invaded the liver or diaphram.

### In vivo *activation of T cells in TCR/RAG mice*

Various studies have shown that initial treatment of mice with a T-cell-activating agent enhances the tumoricidal activity of bispecific antibodies. Two agents that have been used for this purpose are the anti-CD3 antibody 2C11 or the superantigen SEB. In 2C TCR/RAG mice it is also possible, in principle, to use the peptide SIYRYYGL as an activating agent, since it is recognized by CTL 2C in the context of the K$^{b}$-class-I product (Udaka *et al.,* 1996) present in these TCR/RAG mice. To demonstrate the effect of *in vivo* treatment with SIYRYYGL peptide, splenocytes from mice that received i.p. injections of the peptide were assayed directly *ex vivo,* without further culturing, using the L$^{d}$-positive target cell P815, against which clone 2C was originally induced (Kranz *et al.,* 1984). CTL-mediated activity peaked at 1 day after injection, and significant activity could be detected up to 3 days after peptide treatments (Fig. 1*a*). We have shown that the injection of other K$^{b}$-binding peptides not recognized by CTL 2C do not induce T-cell activation in the TCR/RAG mice (Manning *et al.,* 1997).

To assess the feasibility of repeated activation, we compared the *ex vivo* lytic activity from animals that received a single injection of peptide with animals that received 2 injections. The 2 injections were spaced either 1 day or 4 days apart. No significant decline in T-cell cytolytic activity was observed in animals that had received prior injections of the peptide, indicating that mice could be treated with multiple doses of bispecific antibodies following T-cell activation with peptide (Fig. 1*b*).

To confirm that peptide activation of T cells does not itself lead to rejection of KB tumor in a non-specific manner, mice received activating peptide one day before tumor transplantation. The tumors grew equally well in peptide-treated and untreated mice (Fig. 2). This finding further supports earlier observations that the 2C TCR does not cross-react with human tumor antigens. It also

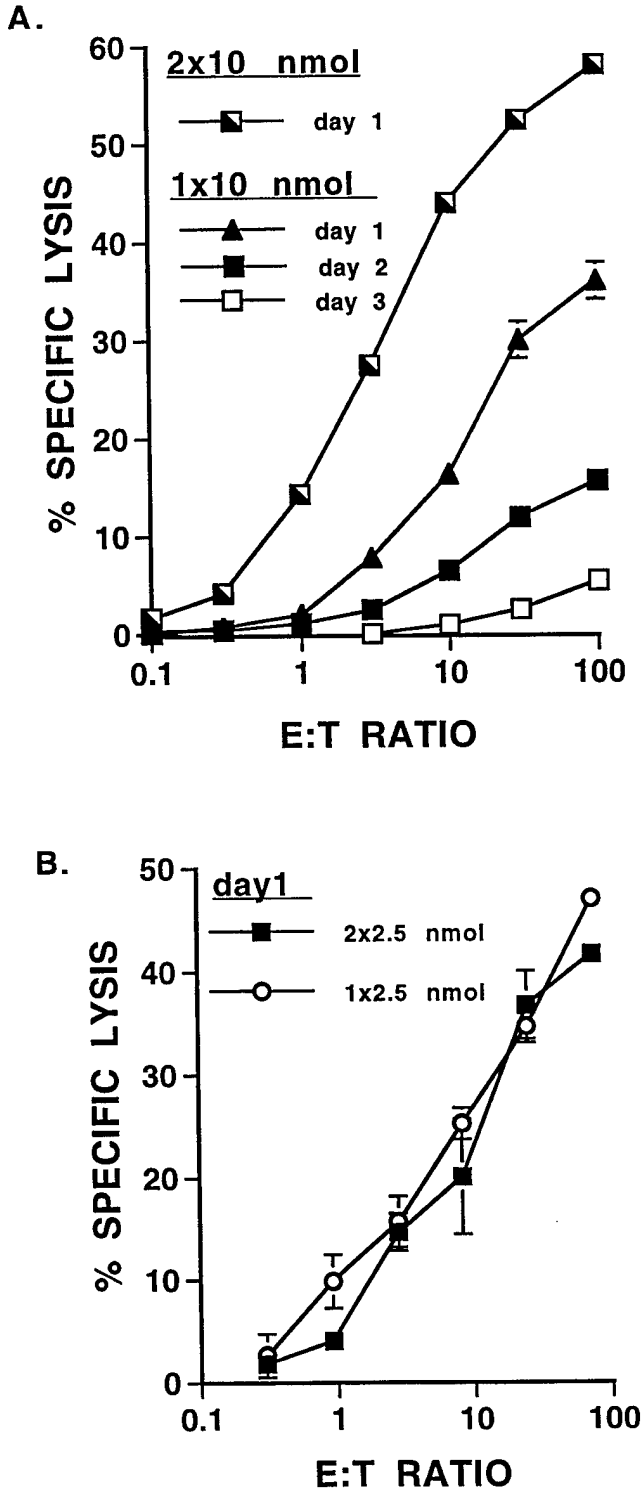


FIGURE 1 – *Ex vivo* cytolytic activity of TCR/RAG splenocytes following treatment with activating peptide. *(a)* Splenocytes were assayed after 1, 2, or 3 days of a single 10-nmol i.p. injection of SIYRYYGL peptide (1 × 10 nmol); or 1 day after 2 injections of 10 nmol peptide on consecutive days (2 × 10 nmol). *(b)* Similar results were observed with a 2.5-nmol dose of peptide: 1 day after a single injection (1 × 2.5 nmol) or 1 day after 2 injections (2 × 2.5 nmol administered on days 1 and 5; analysis on day 6). Splenocytes were assayed at various E:T ratios against $^{51}$Cr-labeled P815 target cells. Standard error bars represent the variation of triplicate wells within the same assay. No cytolytic activity was observed with splenocytes from mice that did not receive peptide (data not shown).

indicates that non-specific inflammation that might be initiated by 2C T cell activation is not capable of eliminating the tumor or detectably slowing its growth.

*T-cell lysis of tumor cells mediated by folate/anti-TCR antibody conjugates*

We have shown that folate conjugates of various anti-TCR antibodies can redirect CTLs to lyse FR-positive mouse tumor cells (Cho *et al.*, 1997; Kranz *et al.*, 1995). In order to determine whether a FR-positive human tumor could likewise be recognized and killed by mouse CTLs in the presence of these conjugates, KB was used as a target cell in cytotoxicity assays. The folate conjugates examined in these experiments included folate/Fab fragments and folate/scFv fragments of the anti-Vβ8 antibody KJ16 (Cho *et al.*, 1997). Both conjugates were capable of mediating the lysis of KB cells by CTL 2C (Fig. 3). The lysis induced in the presence of the folate conjugates was mediated through the folate receptor, as evidenced by the complete inhibition observed in the presence of excess folate (Fig. 3). In addition, unlabeled Fab or scFv fragments did not mediate lysis (data not shown). Finally, spleen cells activated *in vitro* with the peptide SIYRYYGL were also capable of redirecting their cytolytic activity against the KB tumor in the presence of folate conjugates. Together, these results prompted us to evaluate *in vivo* tumoricidal activity of the folate conjugates.

*Bispecific conjugate-dependent rejection of FR$^+$ human tumors by TCR/RAG mice*

The folate conjugates were tested *in vivo* against KB tumor cells that were transplanted s.c. or i.p. The i.p. model was evaluated in addition to the s.c. model, as growth of the KB tumor i.p. provides a more realistic model for adjunctive therapy of disseminated ovarian carcinomas. As indicated above, over 90% of ovarian cancers express the folate receptor, and these levels appear to be similar to the KB tumor cell line (Mathias *et al.*, 1996).

In a preliminary experiment, mice were injected s.c. in the flank with $3 \times 10^6$ tumor cells mixed with PBS (n = 3) or with 20 μg of the folate/Fab conjugate (n = 3) before injection. All mice received activating peptide one day before transplantation. As shown in Figure 4*a*, tumors grew uniformly in all 3 of the control mice, but were completely rejected in 2 of the mice in which tumors were mixed with the folate/Fab conjugate. The other mouse exhibited a 6-week delay in the growth of the tumor. Once established, this tumor grew at a rate similar to tumors from the control mice. Tumors reached 20 mm mean diameter at 36 ± 2.5 days for control and >114 days for the conjugate-treated group ($p < 0.03$). There were no obvious side effects in mice receiving the treatment, including no signs of damage to organs such as the liver and kidney, nor were any histologically abnormalities detected within the tissue that expresses the highest level of folate receptor, the choroid plexus.

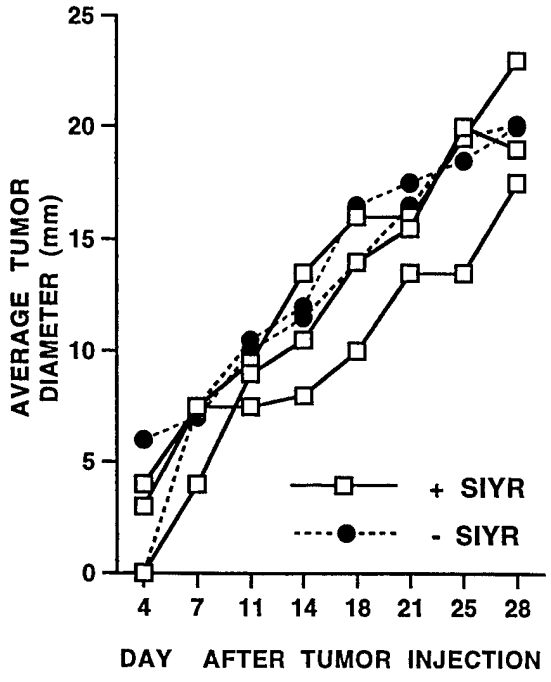


FIGURE 2 – Growth of human tumor xenografts in TCR/RAG mice treated with activating peptide. Mice treated one day previously with 2.5 nmol SIYRYYGL peptide or without peptide, were injected s.c. with KB cells ($1 \times 10^7$ in 200 μl). Vernier calipers were used to take 2 perpendicular measurements of tumors twice weekly. Measurements were averaged to determine mean tumor diameter.

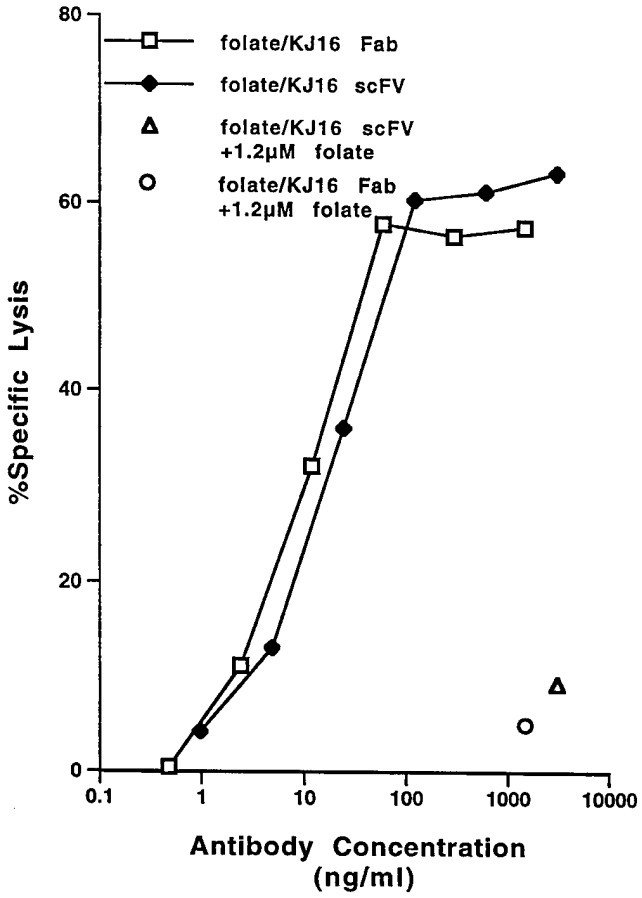


FIGURE 3 – *In vitro*, CTL-mediated lysis of human tumor cells in the presence of folate/anti-TCR conjugates. The mouse CTL clone 2C was used as the effector and the FR positive human KB cell line as the $^{51}$Cr-labeled target ($1 \times 10^4$ cells) at an effector-to-target-cell (E:T) ratio of 67:1. Various concentrations of folate/KJ16 Fab and folate/scFv were added to the cells in triplicate wells in folate-free DMEM + 10% FCS. Excess free folate (1.2 μM) was added to the highest antibody concentration of each conjugate (folate/KJ16 Fab, folate/KJ16 scFv).

Additional experiments were performed to confirm the specificity of the conjugate-mediated rejection and to determine whether it could be extended to the use of folate/scFv conjugates. As shown in Figure 4*b*, tumors grew equally well in mice whether pre-treated with PBS or with 20 μg of unconjugated scFv. Median time to reach mean tumor diameter of 20 mm was 35 days for both. In contrast, mice that received tumors incubated with folate/scFv conjugate exhibited delayed growth of tumors (n = 4; median = 64 days to 20 mm; $p < 0.05$), or complete rejection of the tumor (n = 3; no tumors had developed by the time that they died of non-tumor-related causes on day 59, 99 or 117) (Table I). Mice that received

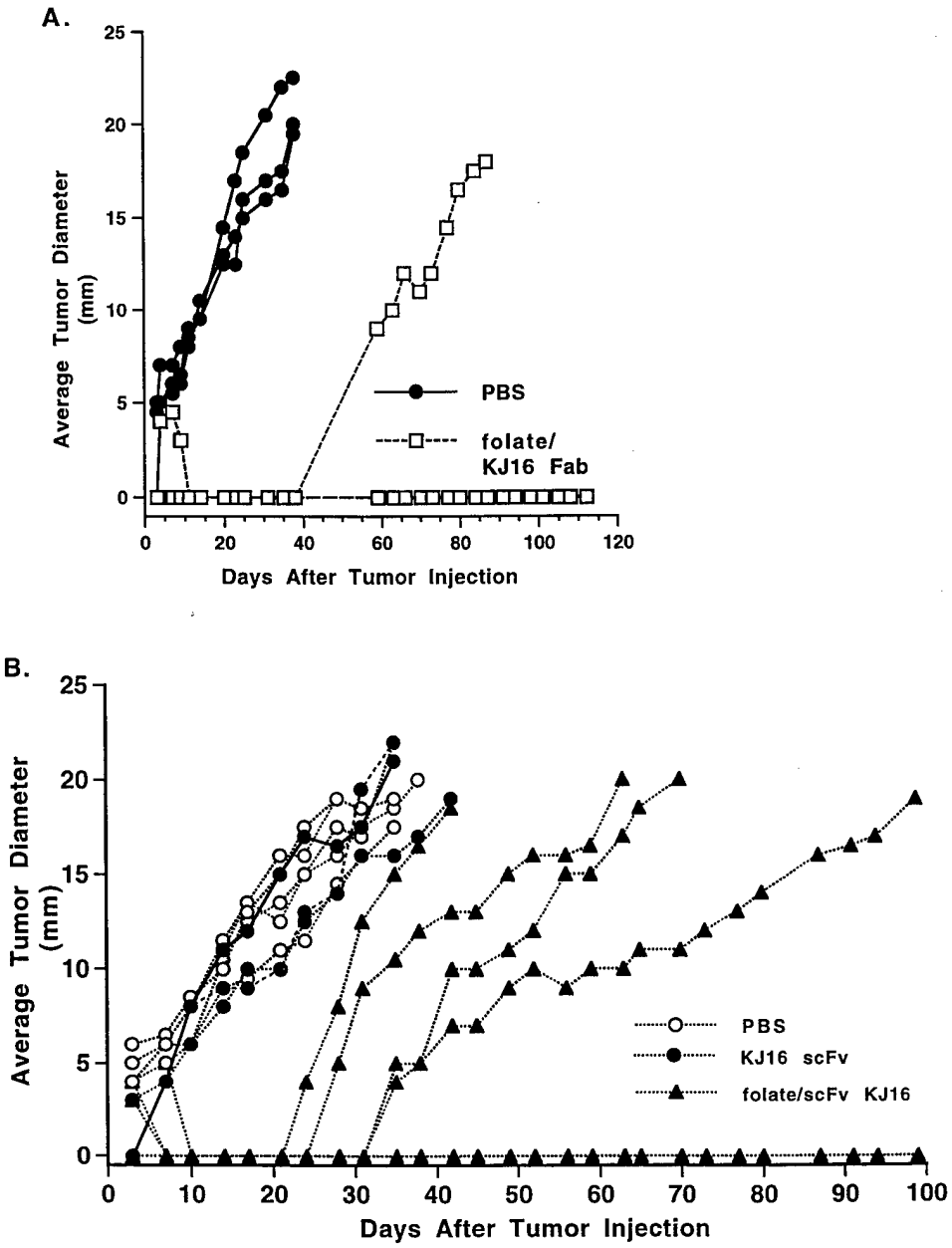


**FIGURE 4** – Effects of folate/antibody conjugates on s.c. growth of KB cells. *(a)* KB cells ($3 \times 10^6$) were mixed with PBS or 20 μg folate/KJ16 Fab fragments, then injected s.c. into TCR/RAG mice (24 hr after administration of 2.5 nmol SIYRYYGL peptide i.p.). Mice received a second peptide dose on day 3 and a second antibody treatment (20 μg; i.p.) on day 4 following tumor injection. Results for 2 mice that completely rejected the tumor are included at the bottom of the graph (open squares; 2 mice had no visible tumor on day 114, when they were killed). *(b)* KB cells ($2.6 \times 10^6$) mixed with PBS, 20 μg scFv-KJ16, 20 μg folate/scFv-KJ16 or 1 μg folate/scFv-KJ16 were injected s.c. into TCR/RAG mice, 24 hr following SIYRYYGL peptide administration (2.5 nmol i.p.). The treatment regime was identical to *(a)*, except that 2 mice received 1 μg folate/scFv treatment after being placed on low-folate chow 3 weeks prior to the tumor injection. This treatment group was added since it had been reported that folate levels in standard mouse chow are considerably higher than in human diet, leading to high serum-folate levels (Mathias *et al.*, 1996). The folate/scFv-KJ16 treatment (▲) slowed tumor growth in 4 mice and caused complete rejection in 3 mice (2 received 1 μg; 1 mouse died at 59 days, apparently of a non-tumor-related cause, while the second mouse showed no sign of a tumor at 117 days; 1 received 20 μg), as shown at the bottom of the graph. Measurements were made twice weekly and mice were killed when the tumor reached a mean diameter of 20 mm. Mean tumor diameter was the average of 2 perpendicular measurements.

s.c. tumors prior to an i.p. injection of the folate/antibody conjugates developed tumors at the same rate as untreated animals (data not shown). This suggested that targeting of the antibody conjugates or T cells to such tumors was not efficient.

In comparison with an s.c. model, the i.p. tumor model more closely resembles advanced human ovarian cancer (Mezzanzanica *et al.*, 1991). Alberts *et al.* (1996) have highlighted the improved efficacy of treating stage-III ovarian cancer with i.p. rather than i.v. cisplatin. We reasoned that a similar approach, using a large-volume i.p. injection of bispecific conjugates might allow for improved delivery of antibody agents directly to the site of the tumor. In this experiment (Fig. 5), each group of mice received $5 \times 10^6$ tumor cells i.p. As we observed in the s.c. model, mice that received tumor cells mixed either with PBS or with 10 μg of

TABLE I – EFFECT OF ANTIBODY TREATMENTS ON s.c. KB TUMOR GROWTH

| Treatment | Number | Median (days) | Mean[1] ± SEM (days) | *p* value[2] |
|---|---|---|---|---|
| Experiment 1 | | | | |
| None | 3 | 38 | 36.0 ± 2.5 | — |
| Folate–Fab (20 μg) | 3 | >114 | >107.3[3] | 0.03 |
| Experiment 2 | | | | |
| None | 3 | 35 | 37.3 ± 2.3 | — |
| scFv | 5 | 35 | 34.8 ± 1.8 | 0.44 |
| Folate–scFv (20 μg) | 5 | 70 | 70.6 ± 9.8 | 0.05 |
| Folate–scFv (1 μg) | 2 | 88 | >117[4] | 0.05 |

[1]Number of days taken to reach a mean tumor diameter of 20 mm.–[2]Comparison of each treatment with the KB control, untreated group.–[3]One mouse had a tumor that reached 20 mm at 94 days, while 2 mice had no visible tumor on day 114, when they were killed.–[4]One mouse died at 59 days, apparently of a non-tumor-related cause, while the second mouse showed no sign of a tumor at 117 days.

unconjugated Fab fragments survived for equal times (median = 24 and 23 days respectively). In contrast, mice that were co-injected with 10 μg of either the Fab or scFv bispecific agents survived for over 70 days, with a single exception ($p < 0.004$). Statistical analyses of these and subsequent i.p. experimental groups are shown in Table II.

Additional groups of mice were included to evaluate the effects of treating animals that did not previously receive T-cell activating agent (*i.e.,* activating peptide) (n = 5) or to evaluate the effects of treating established tumors with i.p. injections of folate/Fab conjugates (n = 5) or folate/scFv conjugates (n = 5). All mice that received tumor cells together with the folate/Fab conjugate, but no activating peptide, exhibited delayed tumor growth, although they eventually succumbed to the tumor (Fig. 5, Table II). Thus, survival of mice that did not receive activating peptide was significantly reduced compared with that of the peptide-treated group ($p < 0.004$). These results postulate an important role for concomitant T-cell activation in enhancing the anti-tumor effects of the bispecific agents.

Mice with previously established peritoneal-cavity tumors that were treated either with the Fab or with scFv conjugates exhibited increased survival times (Fig. 5, Table II). The median survival time of tumor-bearing animals was increased by approximately 85% in each of the 2 treatment groups (Table II). When analyzed as a pooled group receiving bispecific conjugates (Fab or scFv) (n = 10), treated animals exhibited a mean survival time of 38.8 days (SEM = 3.2) with a $p$ value of 0.056 for the difference as compared with untreated animals.

*T-cell infiltration and cytolytic activity*

In an effort to understand the *in vivo* effects of bispecific agents and to capture a "snapshot" of the process of T-cell redirection, peritoneal lavage was performed on mice treated with or without bispecific agent and with or without activating peptide. Flow-cytometric analysis of lavage fluid obtained 3 days following antibody therapy showed that the number of T cells infiltrating the peritoneum was increased approximately 10-fold by bispecific treatment, with or without peptide, as compared with unconjugated Fab treatment (Table III). The cytolytic activity in the peritoneal cavity of the same mice was similarly investigated using the $L^d$-positive cell line P815 as a target. In contrast to the observations on T-cell infiltration, significant cytolytic activity was observed only in those animals that received treatment with the activating peptide prior to bispecific-antibody treatment (Table III). Notably, T cells infiltrating the tumor site of mice previously activated with peptide were able to achieve 10 to 45% direct *ex vivo* cytolysis at a 50:1 E:T ratio, while the T-cell-mediated lysis obtained from mice not treated with peptide was less than 10%. The lysis was clearly due to infiltrating T cells and not other effector cells, since the anti-TCR antibody 1B2 was able to inhibit the activity (Table III). Thus, it appears that bispecific agents used alone can induce infiltration of CTLs to the tumor site and mediate some degree of anti-tumor effect, but the concurrent use of T-cell-activating agents is required for full development of cytolytic activity in the redirected cells.

## DISCUSSION

Pre-clinical studies to evaluate bispecific antibodies that recognize human tumor antigens have required immunodeficient animal models. Typically in these models, the human tumor, activated human effector cells and the bispecific antibodies to be evaluated are each administered to the immunodeficient animal (*i.e.,* nude or SCID mice). Unfortunately, these models do not accurately reflect the most desirable clinical uses of bispecific agents. These uses will include a treatment regimen in which only the antibody agents and an appropriate activating peptide need be administered in order to redirect a patient's own immune system. In this report, we describe the development of a TCR/RAG-$1^{-/-}$ animal model that more closely resembles this clinical scenario in allowing anti-human-tumor-antigen reagents to be evaluated for their ability to mobilize endogenous T cells.

The 2C TCR transgenic system is particularly useful, since there have been many studies to identify the peptides recognized by CTL 2C and to explore the optimal activation requirements of 2C T cells. One of these peptides (SIYRYYGL) can be conveniently used to activate 2C transgenic T cells *in vivo,* thus facilitating a dissection of the role of concurrent T-cell activation in bispecific-antibody based therapies. The SIYRYYGL peptide can, in fact, activate cells in TCR/RAG mice (Fig. 1), and these activated T cells are able to potentiate the *in vivo* effects of the bispecific agents (Table II, Fig. 5). While obviously this specific peptide will not be applicable to human therapy, it now becomes possible to compare the efficacy of antigen-induced activation with activation induced by other more broadly applicable agents such as anti-CD3 and/or anti-CD28 antibodies. These agents, or others that act on the majority of human T cells, are likely to serve the same role as peptide did in the present study.

Analysis of the T cells infiltrating the site of the tumor after peptide activation and bispecific redirection showed that effector CTLs can be localized to the tumor site (Table III). Importantly, these results highlighted a critical role for activation signals in maximizing the efficacy of bispecific antibodies. As shown in Table III, bispecific conjugates were sufficient to localize and retain T cells at the site of the tumor, but in the absence of an activating agent, these cells did not achieve lytic potential. Nevertheless, some efficacy of the bispecific conjugates was observed in animals treated without activating agents (Table II), as manifested by an approximate doubling of mean survival over untreated animals. However, the effect of bispecific conjugates was significantly increased when the T cells were activated prior to treatment (Table II, Fig. 5). These results are interesting in the light of a study using *ex vivo* activated human T cells together with bispecific antibody in patients with advanced ovarian carcinoma (Lamers *et al.,* 1997): this study showed that T cells recovered from peritoneal fluid exhibited relatively low cytolytic activity, but the activity was approximately the same in patients who responded and in those who did not respond to treatment. It is possible that strategies to enhance the cytolytic activity and persistence of such T cells at the tumor site will yield improved responses.

Several factors suggest that the potential efficacy of bispecific agents in humans may be enhanced as compared with their performance in the TCR/RAG system. For example, while TCR/RAG mice express a population of homogenous $CD8^+$ cells, the total number of CTL is less than or equal to the number found in a normal mouse. Perhaps more importantly, these mice do not express $CD4^+$ cells, which have been shown to be important in sustaining T-cell-mediated responses, *i.e.,* the monoclonal population of $CD8^+$ CTL precursor cells in the 2C TCR/RAG mice have achieved their entire anti-tumor effect acting in the absence of

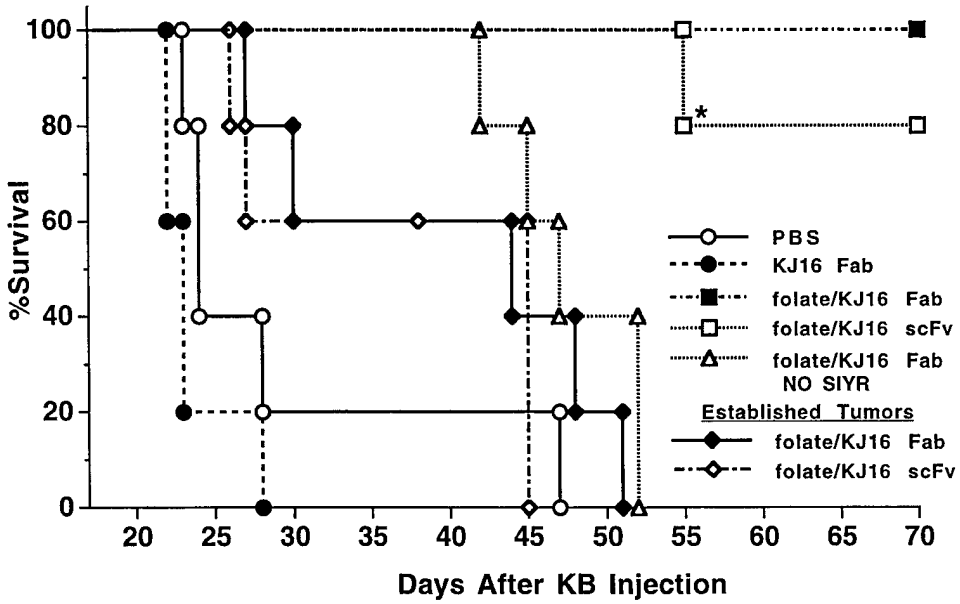


FIGURE 5 – Effects of folate/antibody conjugates on the survival of TCR/RAG mice with peritoneal tumors. KB cells ($5 \times 10^6$) mixed with PBS, 10 μg KJ16 Fab fragments, 10 μg folate/KJ16 Fab fragments, or 10 μg folate/scFv were injected i.p. into TCR/RAG mice (n = 5 per treatment) as a single-cell suspension in 1 ml. Antibodies were administered twice, first mixed with the cells, then 4 days later. For the established groups, antibodies were added on days 2 and 6 after tumor injection (established folate/Fab fragments; established folate/scFv). In all except the NO PEPTIDE treatment group, 2.5 nmol of SIYRYYGL peptide was administered i.p. 24 hr before each antibody injection. The NO PEPTIDE treatment group received 10 μg folate/KJ16 Fab fragments mixed with tumor cells as in experimental group. Mice were monitored daily for signs of illness and were killed when they became moribund. The mouse marked * died on day 55, apparently of a non-tumor-related cause. The time line indicates the treatment regime used in the experiment. "Established" refers to animals that received tumor only on day 0.

TABLE II – EFFECT OF ANTIBODY TREATMENTS ON SURVIVAL OF MICE THAT RECEIVED i.p. KB TUMORS

| Treatment | Number | Median | Mean ± SEM | *p* value[1] |
|---|---|---|---|---|
| None | 5 | 24 | 29.2 ± 4.5 | — |
| Fab | 5 | 23 | 23.6 ± 1.2 | 0.08 |
| Folate–Fab | 5 | >70[2] | >70 | <0.004 |
| Folate–scFv | 5 | >70[3] | >70 | <0.004 |
| Folate–Fab (without activating peptide) | 5 | 47 | 47.6 ± 2.0 | 0.03 |
| Folate–Fab (established) | 5 | 44 | 40.0 ± 4.8 | 0.07 |
| Folate–scFv (established) | 5 | 45 | 37.6 ± 4.5 | 0.23 |

[1]Comparison of each treatment with the KB control, untreated group.–[2]At the time of reporting (day 70), all mice in this treatment group were alive.–[3]At the time of reporting (day 70), 4 mice were alive and 1 died on day 55, apparently of a non-tumor-related cause.

TABLE III – EFFECTS OF FOLATE–ANTIBODY CONJUGATES ON T-CELL INFILTRATION AND CYTOLYTIC ACTIVITY AT THE TUMOR SITE

| Treatment[1] | TCR/RAG mouse | T-cell number[2] (×10⁵) | Lysis[3] −1B2 (%) | Lysis[3] +1B2 (%) |
|---|---|---|---|---|
| KB + Fab | 1 | 7 | 15 | 11 |
| | 2 | 7 | 7 | 5 |
| | 3 | 3 | 9 | 7 |
| | 4 | 3 | 5 | 2 |
| | 5 | 1 | 2 | 3 |
| KB + folate–Fab | 6 | 75 | 1 | 2 |
| | 7 | 27 | 6 | 5 |
| | 8 | 18 | 10 | 5 |
| | 9 | 16 | 10 | 5 |
| | 10 | 8 | 6 | 2 |
| KB + SIYR + folate–Fab | 11 | 50 | 45 | 7 |
| | 12 | 40 | 9 | 2 |
| | 13 | 34 | 31 | 3 |
| | 14 | 32 | 45 | 4 |
| | 15 | 22 | 12 | 4 |

[1]KB cells ($2 \times 10^6$) were injected i.p. on day −2. The following day, 2.5 nmol activating peptide were administered to one group. Antibody treatments (KJ16 Fab or folate–Fab, 1 μg) were injected on day 0.–[2]Three days after antibody treatments, peritoneal lavage was performed to collect peritoneal exudate cells (PECs). Total PECs were counted and examined by flow cytometry to identify the 2C T cells, by means of an anti-Thy 1.2 antibody–phycoerythrin conjugate.–[3]Cytolytic activity of PECs was determined at a 50:1 E:T ratio using $^{51}$Cr-labeled P815 target cells in the presence or absence of the MAb 1B2.

CD4-derived help. Additionally, lysis of human tumor cells by mouse CTLs is less efficient than is lysis by human CTLs, due to interspecies differences in adhesion and accessory molecules (Kranz *et al.*, 1984; and data not shown), and an important role for these molecules in T-cell therapy has been demonstrated by Weijtens *et al.* (1998). Finally, since the T-cell repertoire of the 2C TCR/RAG mice is limited to a single clone, it cannot be expected that the response elicited by bispecific redirection could promote the expansion of tumor-specific T cells and a memory response upon rechallenge. However, in a normal heterogeneous immune system, it is possible that a redirected response against a syngeneic tumor would act to elicit tumor-specific T cells.

The issues raised above support the notion that bispecific antibodies should be tested in both the xenograft system described here and in syngeneic mouse tumor models. In this respect, one of the key advantages of the folate conjugates is that exactly the same conjugates can be compared in syngeneic mouse systems. There are 2 different syngeneic tumors that express the folate receptor: the DBA/2-derived tumor F2-MTX$^r$A (Kranz *et al.*, 1995), and choroid plexus tumors that arise endogenously in an SV40 transgenic mouse line (Patrick *et al.*, 1998; Roy *et al.*, 1998).

The use of the 2C TCR/RAG system also will allow the evaluation of aspects of T-cell trafficking in bispecific-conjugate treated mice. These studies can be conveniently performed, since all the T cells are monoclonal and therefore express the same TCR identifiable by the anti-clonotypic antibody 1B2 (Kranz *et al.*,

1984). Thus, it is now possible to explore the optimal activating and retargeting agents as well as the regimen required to maximize infiltration of T cells into the tumor. This will be particularly important, since our findings have shown that treatment of established solid tumors with folate conjugates will benefit from improvements in this aspect of the therapy. Other studies have found similar problems associated with bispecific-antibody therapy of solid tumors (Bakacs *et al.,* 1995; Mezzanzanica *et al.,* 1991). A more complete understanding of T-cell activation and migration properties (Zhu *et al.,* 1996) should allow the identification of agents that have improved T-cell-modulating properties.

In order to exploit the advantages of the TCR/RAG system, we have developed a human tumor model for $FR^+$ ovarian carcinomas. In the present studies the human tumor line KB, used for many *in vitro* studies with folate-receptor-specific agents, was injected either s.c. or i.p. The i.p. $FR^+$ tumor model can serve to mimic the condition of disseminated peritoneal carcinomatosis, as well as the seeding of the peritoneal cavity that can occur following ovarian tumor resection. Mice with established i.p. tumors exhibited an approximate doubling of survival times after treatment with folate/anti-TCR conjugates (Table II). Our findings using the endogenous brain-tumor model in SV40 transgenic mice also showed that folate/scFv treatment could extend survival by approximately 2-fold *vs.* control mice (Patrick *et al.,* 1998; Roy *et al.,* 1998). We foresee a time when patients with $FR^+$ ovarian or brain tumors might be treated with peritoneal (Bolhuis *et al.,* 1992; Canevari *et al.,* 1995) or intrathecal administration of bispecific folate conjugates in the post-operative period, to address tumor seeding or unresectable tumor remnants.

This study also demonstrates the significant therapeutic potential of a new class of targeting agents that are smaller and easier to produce than conventional bispecific antibodies. These folate/antibody conjugates are capable of efficiently targeting large classes of tumors that bear the high-affinity folate receptor as a tumor marker (Cho *et al.,* 1997; Kranz *et al.,* 1995; Patrick *et al.,* 1998; Roy *et al.,* 1998). In addition, the ease of producing them will allow the study of other folate/antibody conjugates (*e.g.,* folate/anti-CD28) to test their ability to enhance T-cell responses in the TCR/RAG system described here. Finally, it is notable that other tumors, such as the human erbB-$2^+$ tumors BT474 and SKOV3, also readily grow in the TCR/RAG mice. Thus, it will be possible to evaluate other bispecific anti-human-tumor-antigen antibodies in the TCR/RAG system. In the case of the KB line, we found 100% tumor acceptance rate in TCR/RAG mice, providing further evidence that these mice do not exhibit the "leakiness" of SCID mice. Our ultimate goal is to develop a model in which combinations of agents to be used in humans could be tested against human tumors transplanted into animals which, like the patient, possess competent endogenous immune effector cells. The TCR/RAG system described here approaches this goal, although the anti-effector agent remains an anti-mouse-T-cell-receptor antibody. In the future, this aspect can be improved, either by using antibodies that cross-react with human and mouse antigen-receptors or, possibly, by the development of transgenic mice that express the relevant human antigen (*e.g.,* human CD3).

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